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Pharmacological potential of *Baccharis dracunculifolia* in the control of emotional-like comorbidities in chronic pain

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DECLARAÇÃO

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Abstract

In osteoarthritis (OA) the progressive degeneration of articular structures persistently activates nociceptors leading to chronic pain. Chronic pain is often accompanied by the comorbid development of emotional impairments, including anxiety and depression, an effect recently associated to changes in the activation level of microglia in the brain.

Baccharis dracunculifolia DC (Asteraceae) (Bd) is a Brazilian medicinal shrub, popularly known as "Alecrim do Campo", shown to be an important source of active compounds with anti-stress and anti-inflammatory ability.

After assessing its phytochemical profile, *B. dracunculifolia* antioxidant activity was evaluated using several *in vitro* models: free radical scavenging (DPPH method), iron chelating activity (ICA), NO and SO scavenging with quercetin used as a reference standard.

The ability of *B. dracunculifolia* extract in reversing the OA-induced nociceptive and emotional impairments was studied in ovariectomized adult female rats (*Rattus norvegicus, vr. albinus, wistar*) using the kaolin/carrageenan (K/C) model. Four weeks after OA induction, mechanical hyperalgesia was confirmed and the pharmacological treatment started. Control animals (SHAM) were administered PBS while ARTH animals either received PBS or *B. dracunculifolia* 50mg/kg (Bd50) and 100 mg/kg (Bd100), via gavage, daily for five weeks.

At the end of the treatment, anxiety-like behaviour was determined using the Open Field Test (OFT), anhedonia using the sucrose preference test (SPT) and learned helplessness using the forced swimming test (FST). Animals were then euthanized, the brains excised, preserved and sectioned. Activated microglia was stained with IBA-I and quantified in brain slides of target areas (Prefrontal Cortex, amygdala and periaqueductal gray matter)

Our phytochemical analysis showed the Bd extract mainly contains phenolic compounds which is in accordance with its significantly higher activity in scavenging SO radicals. Treatment with Bd extract reversed OA-induced mechanical hyperalgesia and partly reversed anxious and depressive-like behaviour in ARTH animals concomitant to a decrease in the number of brain activated microglia.

Our findings suggest Bd extracts can potentially be used as adjuvants in the management of OA-induced pain and associated emotional comorbidities.

Resumo

Na Osteoartrose (OA), a degeneração progressiva das estruturas articulares ativa os nociceptores, provocando dor crónica. A dor crónica é acompanhada pelo desenvolvimento de comorbidades emocionais, incluindo ansiedade e depressão, um efeito recentemente associado a alterações dos níveis de ativação das células da microglia no encéfalo.

Baccharis dracunculifolia DC (Asteraceae) (Bd) é um arbusto medicinal da flora brasileira e popularmente conhecido como "Alecrim do Campo", caracterizada como uma importante fonte de compostos ativos com capacidade antisstress e anti-inflamatória.

Após a análise fitoquímica, a atividade antioxidante de *B. dracunculifolia* foi avaliada utilizando vários modelos *in vitro*: eliminação de radicais livres (método de DPPH), atividade de quelação de ferro (ICA), eliminação de NO e SO, usando a quercetina como padrão referencia.

A capacidade do extrato de *B. dracunculifolia* na reversão dos comportamentos nociceptivos e emocionais foi estudada em ratos fêmeas adultas ovariectomizadas (*Rattus norvegicus, vr. abinus, wistar*) com um modelo de caulina/carragenina (K/C). Quatro semanas após indução de OA, a hiperalgesia mecânica foi confirmada e iniciou-se o tratamento farmacológico. Aos animais controlo (SHAM) foi administrado PBS, enquanto os animais ARTH receberam PBS ou extrato de *B.dracunculifolia* em doses de 50mg/kg (Bd50) e 100 mg/kg (Bd100), via gavagem, diariamente, por cinco semanas.

No final do tratamento, o comportamento ansioso foi determinado utilizando o Teste de Campo Aberto (OFT), a anedonia usando o Teste de Preferência de Sacarose (SPT) e o desamparo aprendido com o Teste de Natação Forçada (FST). Os animais foram eutanasiados, os cérebros foram excisados, preservados e seccionados. A microglia ativada foi corada com IBA-I e quantificada num subconjunto de lâminas cerebrais contendo as áreas alvo (Cortex Prefrontal, Amigdala e Substância Cincenza Pariequeductal).

A nossa análise fitoquímica mostrou que o extrato de Bd é composto principalmente por compostos fenólicos, o que está de acordo com a elevada atividade de sequestro de radicais SO. O tratamento com o extrato de Bd reverteu a hiperalgesia mecânica induzida por OA e reverteu parcialmente o comportamento ansioso e depressivo dos animais. Concomitantemente, o tratamento com Bd diminuiu o número de microglia ativada no encéfalo dos animais ARTH.

Os nossos resultados sugerem que os extratos Bd podem ser usados como adjuvantes no tratamento da dor induzida pela OA e comorbidades emocionais associadas.

Abbreviations

ACC – anterior cingulate cortex

ANOVA1W – ANOVA one-way

AMY – amygdala

ARTH – Artritic group

Bd – *Baccharis dracunculifolia*

Bd100 – Treatment group of 100mg/Kg of *Baccharis dracunculifolia*

Bd50 - Treatment group of 50mg/Kg of *Baccharis dracunculifolia*

BHA – 2 or 3 tert-butyl-4-methoxyphenol

BHT – di-tert-butylmethylphenol

CQAs - caffeoylquinic acids

CC – Cingulate Cortex

CNS - central nervous system

cVLM – caudal Ventrolateral Medulla

DLPAG – Dorsolateral periaquedutal grey matter

DMM – Destabilization of the medial meniscus

DMSO – Dimethyl Sulfoxide

DPPH – 2,2-diphenyl-1-pocryl-hydrazyl

DRt – Dorsal reticular nucleus

EC50 – Efficientt concentration of 50%

FST – Forced swimming test

GAD – Generalized Anxiety Disorder

Gf – grams of force

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

ICA – Iron chelating activity

IBA-I – Ionized calcium-Binding Adapter molecule 1

ILC – Infralimbic Cortex

i.p. – intraperitoneal injection

K/C - kaolin/carrageenan

LPAG – Lateral periaquedutal grey matter

LWT - limb withdrawal threshold

MCI – Mild Cognitive Impairment

MDD – Major Depressive Disorder

MIA – monoiodoacetate

NON-N – non-nociceptive neurones

NS – nociceptive specific neurons

NSAIDs – Nonsteroidal Anti-Inflammatory Drugs

OA – osteoarthritis

OFT – Open Field test

PAG – periaquedutal grey matter

PAM - pressure application measurement

PBS – Phosphate buffer solution

PFA – paraformaldehyde

PFC – prefrontal córtex

PLC – Pre Limbic Cortex

ROS – Reactive Oxigen Species

RVM – Rostral Ventromedial Medulla

SEM – standard error of the mean

SPT – Sucrose preference test

STT – Spinothalamic tract

S1 - primary somatosensory cortex

S2 - secondary somatosensory cortex

UV – Ultra Violet

VCD – 4-Vinycyclohexene diepoxide

VLPAG - ventrolateral periaqueductal gray

WHO – World Health Organization

WDR – wide-dynamic range neurons

1.1 Importance of medicinal plants for humanity

The use of natural products with therapeutic properties is as old as human civilization and for a long time mineral, vegetable and animal products were the main sources of medicines (Zanin *et al.*, 2012).

The Industrial Revolution and the development of organic chemistry increased the preference for synthetic products mainly because pure compounds were easier to obtain and to alter structurally to produce potentially more active and safer drugs (Rates, 2001). In addition, throughout the development of human culture, the use of natural products has had religious and/or exoteric significance, with different views on the concepts of health and disease within each culture. This approach was thus against the new lifestyle of industrialized western societies, where medicines obtained from natural resources were considered an option for people of low income and education or simply as religious superstition, and thus, with no pharmacological value (Rates, 2001, Andrade *et al.*, 2007).

However, in recent years the interest in alternative therapies and in the therapeutic use of natural products has grown, especially in plant derived products (Andrade *et al.*, 2007). This interest in medications of plant origin is due to the abusive and/or incorrect use of certain drugs in conventional medicine leading to lack of results or unwanted side effects. Another problem is the high percentage of the world population that does not have access to conventional medicine and pharmacological treatments (Rates, 2001).

In addition, there are several reasons for researching medicinal plants, including gaining knowledge about the medicinal potential of a great diversity of native plants, to establish a rational basis for the medicinal use of certain plant species, the development of herbal medicines that are low cost but exhibit relevant activity, to discover new drug prototypes, and to obtain information about traditional drugs (Almeida *et al.*, 2006; Zanin *et al.*, 2012).

Some Eastern countries, such as China and India, have a well-established herbal drug industry and Latin American countries have been investing in herbal research programs and the standardization and regulation of plant therapeutic products, motivating the pharmaceutical industry to target research into the development of herbal medicines (Rates, 2001; Zanin *et al.*, 2012).

1.2 *Bacharis dracunculifolia*

The genus *Baccharis* is composed of more than 500 species distributed mainly in the tropical areas of South America, mainly in southeastern Brazil, Argentina, Paraguay, Uruguay, Colombia, Chile, Mexico and Bolivia. Many are widely used in folk medicine, both for the treatment and prevention of various diseases (Abad & Bermejo, 2007; Massignani *et al.*, 2009; Guimarães *et al.*, 2012; Rodrigues *et al.*, 2009).

The main representative of the genus is *B. trimera*, widely found in South America and popularly known as "carqueja" or "carqueja-amarga". It is mainly used as digestive aid, diuretic, hepatoprotective, anti-inflammatory, and antihypertensive agent (Ramos Campos *et al.*, 2016). Another very common species is *B. articulata*, known as "erva-doce", traditionally used in folk medicine as a digestive, diuretic, tonic, and antipyretic aid, organic weakness and anemia (Ramos Campos *et al.*, 2016). In Paraguay, the air infusion of *B. articulata* is used as an antidiabetic agent (Abad & Bermejo, 2007; Ramos Campos *et al.*, 2016). Also *B. illinita*, known as "chá-ventura" or "erva-milagrosa", is commonly used by the population as an anti-inflammatory (including topical application), gastroprotective and anti-infective agent (Ramos Campos *et al.*, 2016). According to Ramos Campos *et al.* (2016) only 30% of all *Baccharis* species were so far investigated at the chemical or biological level. Due to the ethnobotanical importance of this genus in traditional medicine and its economic impact, studies on new species of *Baccharis* are urgently needed.

The present research work focuses on the species *Baccharis dracunculifolia* DC (Asteraceae). *B. dracunculifolia* (Fig. 1) is a medicinal shrub originating from the Brazilian flora, and popularly known as "Alecrim do campo" (Guimarães *et al.*, 2012). This medicinal plant is the main botanical source of resin and chemical components of the Brazilian propolis, or green propolis (Guimarães *et al.*, 2012). In traditional medicine, it is used as an anti-inflammatory and for gastric protection (Ramos Campos *et al.*, 2016; Guimarães *et al.*, 2012).

In recent years, there has been a growing interest in studying the chemical profile and biological activity of Brazilian green propolis and its main botanical source, *B. dracunculifolia* (Cestari *et al.*, 2011; Guimarães *et al.*, 2012). Although the composition of green propolis is more complex and unpredictable, its pharmacological potentialities are related to compounds known as constituents of the plant *B. dracunculifolia* (Cestari *et al.*, 2011).

Several studies have identified *B. dracunculifolia* as an important source of antimicrobially active compounds (Filho *et al.*, 2008), protective capacity against gastric ulcers, reduced gastric juice

and increased stomach pH (Lemos *et al.*, 2007; Massignani *et al.*, 2009), hepatoprotective capacity (Rezende *et al.*, 2014), antigenotoxic and antimutagenic capacity, when used in low doses (Andrade *et al.*, 2008; Murani *et al.*, 2008; Rodrigues *et al.*, 2009), immunomodulator (Missima *et al.*, 2007), anti-stress capability (Missima & Sforcin, 2008), anti-inflammatory capacity (Paulino *et al.*, 2008; Cestari *et al.*, 2011) and free radical scavenging (Nakanishi*et al.*, 2003; Guimarães *et al.*, 2012).

Figure 1. Photography of the aerial parts of the plant species *Baccharis dracunculifolia* (Photography by Débora Santos).

1.3 Oxidative stress

Several metabolic processes of living organisms and/or environmental stresses can generate reactive species, such as free radicals and mainly reactive oxygen species (ROS) (Mishra *et al.*, 2012). Free radicals are atoms or molecules that, during metabolic processes are continuously produced and act as mediators in the transfer of electrons in various biochemical reactions (Uttara *et al.*, 2009). Reactive oxygen species (ROS) are partially reduced forms of atmospheric oxygen (O_2).

In normal cells, there is an adequate balance between antioxidants and pro-oxidants. However, this balance can be altered when the production of oxygen species is increased considerably or when

the levels of antioxidants are decreased and this imbalance is termed oxidative stress (Gülçin, 2012).

ROS display different roles in the body and are involved in energy production, phagocytosis, regulation of cell growth, intercellular signaling and the synthesis of important biological metabolites (Gülçin, 2012). On the other hand, its excess has a negative impact, such as DNA damage, altering the structure of proteins and cellular organelles and consequently its impairing their function. ROS negative impact is often associated to pathologies such as cancer, early aging, cardiovascular diseases, degenerative and neurological disorders, among others (Gülçin, 2012). In order to counteract these effects, organisms produce substances capable of regenerating or preventing oxidative damage, playing an antioxidant role (Gülçin, 2012; Manke *et al.*, 2013). In addition to these natural substances, it is possible to obtain other substances capable of sequestering free radicals through external sources such as food and beverages (Gülçin, 2012; Manke *et al.*, 2013).

The amount of antioxidants produced by the body are however insufficient to counteract the effects of the free radicals produced thus suffering degenerative actions (Sarandol *et al.*, 2007; Manke *et al.*, 2013). Oxidative stress has, per example, been implicated in several neuropsychiatric diseases, including major depressive disorder (Lindqvist *et al.*, 2007).

1.4 Antioxidants as protectors against oxidative stress

Antioxidant compounds may be defined as substances which, when present in small concentrations relative to the oxidizable substrate, are able to retard or even substantially inhibit the oxidation of the substrate (Niki, 2010; Silva *et al.*, 2011; Gülçin, 2012). There are two main categories of antioxidants, those of natural origin and those of synthetic origin (Cheung *et al.*, 2003).

In the food industry, the practice of adding antioxidants of synthetic nature, such as 2- or 3-tert-butyl-4-methoxyphenol (BHA) and di-tert-butylmethylphenol (BHT), has been common for food preservation and increased shelf life (Contini *et al.*, 2008). However, carcinogenic properties have been described as a consequence of the use of these types of antioxidants (Aksoy *et al.*, 2016). In

order to eliminate this problem, some researchers defend the substitution of these antioxidants by natural antioxidants, such as quercetin (Contini *et al.*, 2008; Dudonné *et al.*, 2009).

Natural antioxidants are divided into two major groups: the enzymatic and the non-enzymatic (Fig. 2). Enzymatic antioxidants include the major antioxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase. Some examples of non-enzymatic antioxidants include vitamin C, water-soluble phenolic compounds and liposoluble compounds (vitamin E and carotenoids) (Ratnam *et al.*, 2006; Ndhlala *et al.*, 2010).

Epidemiological studies indicate there is a direct relationship between the consumption of antioxidant-rich products and the reduction of morbidity and mortality (Huang *et al.*, 2005). Evidence implicating oxidative stress in the development of various diseases and imbalances leads to recognition of the role of antioxidants in preserving human health (Niki, 2010).

Medicinal plants are rarely employed in traditional medicine as antioxidants, yet their therapeutic properties were considered, in part, because of their ability to eliminate free radicals (Desmarchelier *et al.*, 1999; Niki, 2010). Free radicals may be involved in many diseases, such as inflammatory diseases and gastric ulcers, where antioxidants can act to lessen the oxidative stress that occurs in these cells (Desmarchelier *et al.*, 1999; Niki, 2010). The pharmacological capacity of medicinal plants has been proven, demonstrating they are sources of phenolic compounds that can counteract the negative effects of ROS, and be isolated and used as constituents of functional formulations (Silva *et al.*, 2008; Misan *et al.*, 2011; Lee *et al.*, 2013).

Current studies on free radicals corroborate the theory that antioxidant-rich foods play an essential role in preventing cardiovascular disease, cancer and neurodegenerative diseases, as well as inflammation and problems caused by aging cells (Misan *et al.*, 2011). The elimination of ROS and/or oxidative damage mediated by ROS may also play a key role in the prevention of cancer metastasis (Gomes de Melo *et al.*, 2010; Lee *et al.*, 2013). Unlike cytotoxic agents that cause damage to tumor cells, antioxidants act to prevent the onset of cancer during carcinogenesis, and are generally beneficial to cells (Gomes de Melo *et al.*, 2010; Misan *et al.*, 2011; Lee *et al.*, 2013).

Medicinal plants bring several benefits to human health, in part due to the presence of phenolic compounds. Phenolic compounds comprise one of the largest groups of metabolites present in plants and several beneficial properties have been attributed to these dietary compounds, including antioxidant, anti-inflammatory, and anti-carcinogenic effects (Shukla & Gupta, 2010).

Structurally, phenolic compounds have an aromatic ring with one or more hydroxyl substitutes, including their functional dervivates. The aromatic feature and highly conjugated system with multiple hydroxyl groups make these compounds good electron or hydrogen atom donors, neutralizing free radicals and other reactive oxygen species (Zhang & Tsao, 2016).

The mode of the antioxidant activity of phenolics can be based either on hydrogen atom transfer or single electron transfer by proton transfer. However, the antioxidant potential of a particular phenolic compound primarily depends on the number and position of hydroxyl groups in the molecule (Soobrattee *et al.*, 2005; Zhang & Tsao, 2016).

Degree of hydroxylation also affects the antioxidant activity. Longer distance separating the carbonyl group and the aromatic ring of a phenolic acid seem to increase the antioxidant activity. In addition, increased number of hydroxyl aromatic rings such as flavonoids have been shown higher antioxidant activity compared with phenolic acids (Zhang & Tsao, 2016)

Several beneficial properties have been attributed to these dietary compounds, including antioxidant, anti-inflammatory, and anti-carcinogenic effects. Thus, it is necessary to find new sources of phenolic compounds with therapeutic properties (Shukla & Gupta, 2010). Medicinal plants used in folk medicine have been described to possess high levels of these compounds, consequently, there's a need for increasing knowledge on these plants with the goal of identifying new bioactive compounds.

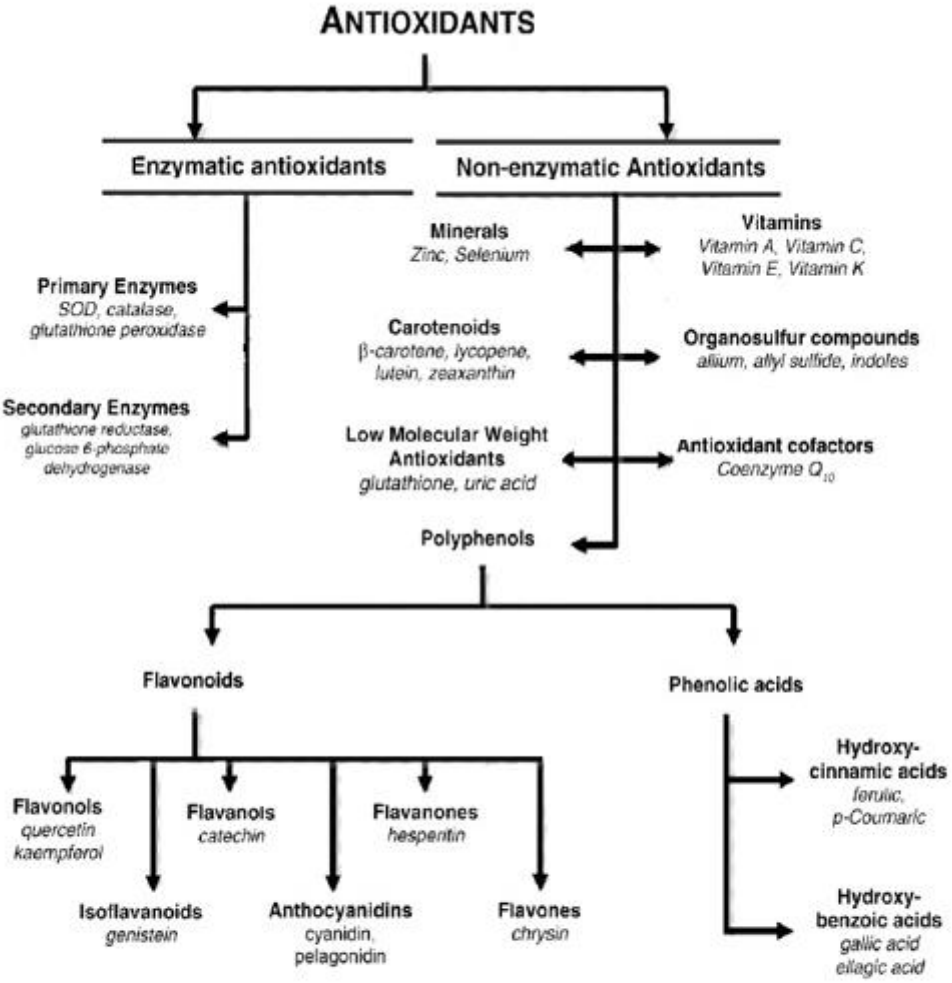

Figure 2. Classification of antioxidants. Some non-enzymatic antioxidants like uric acid, vitamin E, glutathione and CoQ10 are synthesized in the human body but can also be absorbed from dietary sources. Polyphenols are the major class of antioxidants absorbed from the diet (adapted from Ratnam et al., 2006).

1.5 Osteoarthritis

Osteoarthritis (OA) is one of the most common forms of arthritis and the most prevalent disability among the elderly (Van der Kraan *et al.*, 2016). Estimates show it affects about 40 million people in Europe being the 4th disease more common in women and the 8th in men in developed countries (Lawrence *et al.*, 2008; Rutjes *et al.*, 2012; Van der Kraan *et al.*, 2016).

OA is a degenerative disease that causes changes in various tissues inside and in the neighboring areas of joints and whose cause is not fully understood (Van der Kraan *et al.*, 2016). OA causes structural changes including thickness loss of articular cartilage, fibrillation, loss of joint cartilage,

formation of osteophytes, changes in subchondral bone plaque, synovitis and fibrosis in the synovium or capsule (Maldonado and Nam, 2013; Van der Kraan *et al.*, 2016).

This disease can affect several parts of the body, but it is more common in the knee, hip, spine and hand (Cho *et al.*, 2015; Van der Kraan *et al.*, 2016). Clinically it is characterized by joint pain, movement limitation, sensitivity, stiffness, crepitation and various degrees of inflammation (Houard *et al.*, 2013; Van der Kraan *et al.*, 2016).

It is difficult to provide exact numbers on the incidence and prevalence of the disease since radiographic abnormalities do not always correlate with the degree of joint pain reported by patients and vice versa (Van der Kraan *et al.*, 2016). In addition, OA is a slow progressing disease relatively infrequent in people under the age of 40, but increasing with ageing (Deshpande *et al.*, 2016; Van der Kraan *et al.*, 2016). Under the age of 45, women are less affected than men, but this gender difference reverses afterwards (Deshpande *et al.*, 2016; Van der Kraan *et al.*, 2016).

Currently there are no therapeutic solutions available beyond pain control and joint replacement (Van der Kraan *et al.*, 2016). Non-steroidal anti-inflammatory drugs are the most commonly prescribed agents for this condition, however these have side effects, causing frequent severe gastrointestinal and adverse cardiovascular events (Rutjes *et al.*, 2012; Van der Kraan *et al.*, 2016) Another treatment solution is viscosupplementation with hyaluronic acid but the benefits of this treatment are minimal and may cause damage to the knee (Rutjes *et al.*, 2012).

Although aging contributes significantly to cartilage degeneration, other important risk factors include stress, trauma, obesity, metabolic syndrome and genetic predisposition (Houard *et al.*, 2013). It is predicted that the number of individuals with OA will continue to increase in the next decades (Houard *et al.*, 2013).

1.5.1 Animals and experimental OA

Several animal models of osteoarthritis have been developed in an attempt to mimic all aspects of human disease (Bendele, 2001). The available animal models of OA include several species, such as mice, rats, guinea pigs, syrian hamsters, primates, dogs, rabbits (Bendele, 2001), sheep, goats and horses (Gregory *et al.*, 2012).

Some of these OA animal models were used for testing potential antiarthritic and disease modifying agents (Gregory *et al.*, 2012). These agents are currently used in the treatment of patients with OA (Gregory *et al.*, 2012). Studies using rats as animal models are performed based on clinical

disorders and genetic and environmental stimuli, increasing the reliability of these systems to predict drug responses in humans (Aitman *et al.*, 2008). However, human clinical documentation of efficacy (except for symptomatic relief) is deficient due to the difficulties in monitoring and controlling the progression of the disease. As most models have been extensively described in the literature, their relevance to human disease is not based on the history of predictability of drug-induced modification of disease progression, but histopathological similarities to human diseases (Bendele, 2001).

In the present work, the animal species selected to evaluate the pharmacological potential of Bd was the rat although the mouse is one of the most studied experimental animals in biomedical research and provides models for genetic diseases (Bihoreau *et al.*, 1997). In particular, many pure mice strains have been developed for monogenic or multifactorial diseases (Bihoreau *et al.*, 1997).

The laboratory rat (*Rattus norvegicus*) is used as a model animal for physiology, pharmacology, toxicology, nutrition, behavior, immunology and neoplasia studies (Aitman *et al.*, 2008). The advantages of this model are its size, ease of handling and reproductive characteristics, remaining the preferred choice for most of these fields throughout the twentieth century (Aitman *et al.*, 2008).

In rodents, experimental OA can be induced in several ways: mechanically, inducing joint instability through partial meniscectomy combined with transection of collateral ligaments and/or cruciate ligaments (Neugebauer *et al.*, 2007); chemically, through intra-articular injection of irritants and tendon damage compounds and, genetically through the overexpression of mediators such as IL1B (Little and Zaki, 2012).

In the present study, the induction of OA was performed through the intra-articular injection of a solution of kaolin and carrageenan into the synovial cavity of the right knee joint (Pinto-Ribeiro *et al.*, 2008). In this model, the disease develops within a few hours and lasts for several weeks, causing cartilage damage, inflammation of the synovium and exudate of synovial fluid (Neugebauer *et al.*, 2007). This model leads to the gradual degeneration of the articular structures, such as the medial femoral plateaux, promotes increased subchondral bone volume and decreased bone marrow area and formation of subchondral cyst (Amorim *et al.*, 2014).

1.6 Pain

Pain is one of the main reasons for seeking medical advice worldwide (Katz, 2002). According to the International Association for the Study of Pain (IASP), pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Loeser & Treede, 2008).

Pain is a subjective experience, varying substantially from person to person as it is influenced by past experiences, self-expectations about pain, and cultural past (Koyama *et al.*, 2005). In addition, pain is not always associated with an identifiable pathology, since in many cases the pain sensation prevails even in the absence of tissue damage or any other identifiable cause (Savage *et al.*, 2008).

Pain can be divided into acute pain and chronic pain (Loeser & Melzack, 1999). Acute pain is caused by a body injury that activates local nociceptive receptors (Loeser & Melzack, 1999) evoking a first response comprising a protective motor reaction, intended to immediately stop contact with the source of pain (Millan, 2002). Chronic pain is persistent or recurrent pain that lasts for 3 to 6 months without an identifiable cause or long after the original cause has been treated (Johannes *et al.*, 2010; Treede *et al.*, 2015).

1.6.1 Nociception and pathophysiology of pain

Nociception is the encoding of a noxious stimulus, an actual or potential tissue damaging event and its transduction into electric signals (Merighi & Frias, 2016). Nociception becomes unique because individual primary sensory neurons of the 'pain pathway' have the remarkable ability to detect and respond to a wide variety of stimuli, whether chemical, physical or thermal, noxious or innocuous (Julius & Basbaum, 2001).

Noxious stimuli are detected through nerve endings located throughout the body (skin and internal organs), that represent the first element of a polyneuronal chain leading to the perception of pain (Merighi & Frias, 2016).

Upon noxious peripheral stimulation, the nociceptors (sensory fibres – primary afferents) are activated and the nociceptive input is transduced and transmitted to neurones in the superficial dorsal horn of the spinal cord (Basbaum *et al.*, 2009; Merighi & Frias, 2016). Nociceptors are extremely heterogeneous differing in terms of: type of neurotransmitters released; expression of receptors and ion channels, conduction velocity; response properties to noxious stimulus; response

to inflammatory mediators, injury and/or diseases (Stucky *et al.*, 2001; Basbaum *et al.*, 2009; Merighi & Frias, 2016).

First-order neurons are classified into three major groups, according to their diameter, degree of myelination and conduction velocity (Woolf & Ma, 2007; Basbaum *et al.*, 2009):

1. Aβ-fibers are fibers of large diameter (6 to 12 μm), myelinated and fast conducting, responsible for innocuous sensations
2. Aδ-fibers: they are of average diameter (1 to 5 μm), myelinated. Its conduction velocity is intermediate, modulating the first phase of pain: more acute or similar to Stitch.
3. C-fibers are fibers of small diameter (0.2 to 1.5 μm), non-myelinated fibers and slow conduction velocity, responsible for the second phase of pain or diffuse pain.

In the absence of tissue or nervous damage the Aβ-fibers only transmit information regarding innocuous stimuli, such as touch, vibration and pressure (Marchand, 2008). Typically, nociceptive information is transmitted by type C- and Aδ-fibers (Marchand, 2008).

Aδ-fibers are responsible for the first phase of the pain, fast and strong, of the sting or sting type and are sensitive to intense mechanical stimuli (Basbaum *et al.*, 2009). C-fibers produce a second phase of more diffuse and persistent pain and form, on the periphery, receptors for thermal and/or mechanical stimuli. There are also polymodal type C-fibers that respond to mechanical, thermal and chemical stimuli (Basbaum *et al.*, 2009).

In a simplified way, pain transmission can be considered a 3-stage chain process, with the first order neurons located in the periphery transmitting information to the spinal cord, the second order neurons ascending through the spinal cord synapsing at several levels in the brain and the third order neurons synapsing to the cerebral cortex and thalamus where the sensation of pain is perceived (Messlinger, 1997; D'Mello & Dickenson, 2008; Merighi & Frias, 2016).

The dorsal horn of the spinal cord plays an important role in transmitting information from nociceptive primary afferent neurons to the brain; however, our knowledge of its neuronal and synaptic organization is still limited (Todd, 2002).

The gray matter of the spinal cord is divided into ten laminae (Fig. 3), according to their characteristics. Laminae I-VI constitute the dorsal horn, laminae VII to IX the intermediate zone and the ventral horn, and the lamina X is the zone that surrounds the central channel (Diaz & Morales, 2016).

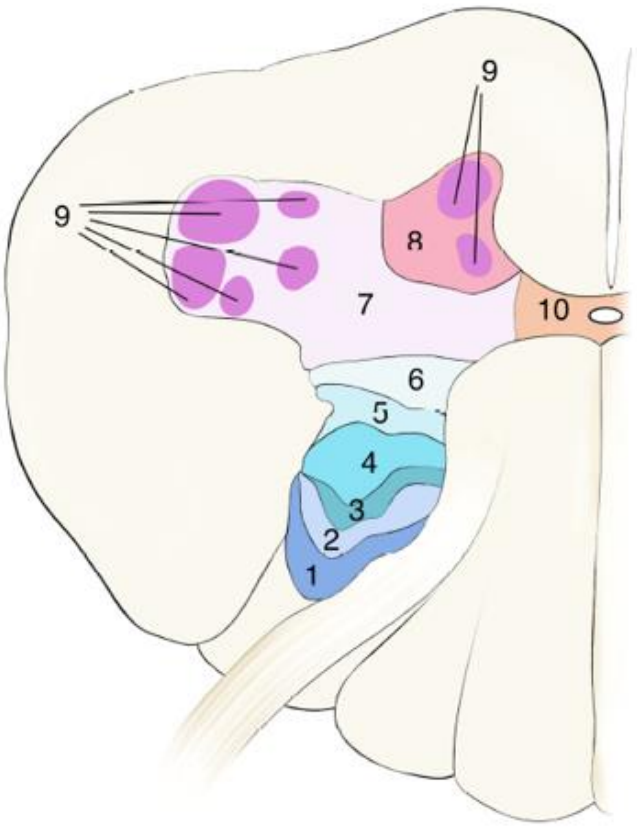

Figure 3. Representation of the division of the gray matter in multiple layers in a cervical spinal cord transverse section. 1: lamina 1 (nucleus marginalis); 2: lamina 2 (substantia gelatinous); 3: lamina 3; 4: lamina 4; 5: lamina 5; 6: lamina 6; 7: lamina 7; 8: lamina 8; 9: lamina 9 ; 10: lamina 10 (Diaz & Morales, 2016).

It has subsequently been found that the various types of primary afferents are not evenly distributed across the dorsal horn laminae. High-threshold unmyelinated C-type nociceptor fibres and thinly myelinated Aδ-type nociceptor fibres transmit nociceptive signals mainly to neurons in spinal lamina I and outer lamina II, whereas low-threshold Aβ-type fibres transmit innocuous touch signals and synapse onto neurons in deeper spinal laminae, particularly lamina III (Fig. 4) (Prescott *et al.*, 2014; Kuner & Flor, 2017).

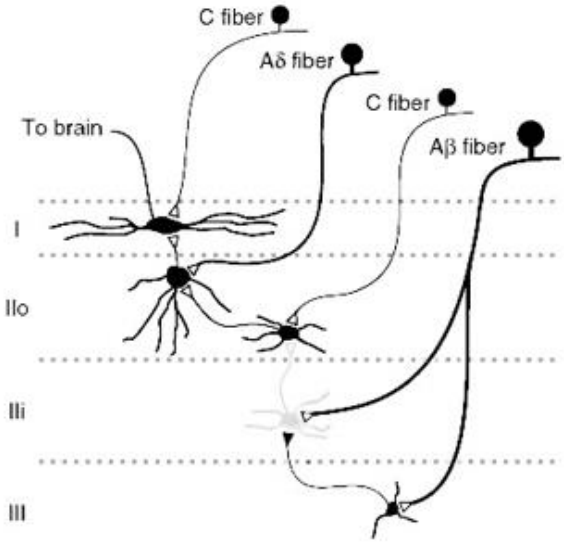

Figure 4. Schematic representation of nociceptor projections to the spinal cord (adapted from Prescott et al, 2014).

There are several types of neuronal cells in the spinal cord that receive projections of primary afferents that can be classified as (i) non-nociceptive (NON-N) when they only respond to touch and receive stimuli mainly from Aβ-fibers, (ii) specific nociceptive neurons (NS), which respond exclusively to noxious stimulation located mainly in the superficial layer of lamina I and receiving projections of the Aδ- and C-fibers and wide-dynamic range (WDR), which receive input from all fiber types and respond to various modalities of stimuli such as mechanical, thermal and chemical stimuli innocuous or noxic (D'Mello & Dickenson, 2008).

Projection neurons have long axons that ascend into the ascending tracts and transmit the sensitive information from the dorsal horn of the spinal cord to higher levels of the neuroaxis, namely to the brainstem and thalamus (Schweinhardt & Bushnell, 2010).

The spinothalamic pathway is traditionally considered the main nociceptive pathway (Merighi *et al.*, 2008). Spinothalamic tract (STT) neurons are both nociceptive specific and WDR neurons (Merighi *et al.*, 2008). Schematically, neurons of the STT localized in lamina I and in laminae IV–VI, especially at the base of the medial aspect of dorsal horn, ascend in the ventrolateral funiculus and terminate in the lateral thalamus (Marchand, 2008; Merighi *et al.*, 2008). This pathway is mostly responsible for the sensory and discriminative aspects of noxious stimuli and is responsible for the motivational and emotional aspects of pain, as well as the escape reaction to acute pain (Marchand, 2008; Merighi *et al.*, 2008).

Ascending spinal pathways are involved in the transmission of nociceptive information to higher centers through five main tracts:

1. The spinothalamic tract
2. The spinoreticular tract
3. The spinomesencephalic tract
4. The cervicothalamic tract
5. The spinohypothalamic tract

Other pain-related ascending pathways are less well characterized and, probably, less critical for sensory-discriminative aspects of pain, but are, nonetheless, important for the general dimension of pain and pain control (Merighi *et al.*, 2008).

The spinoreticular tract originates from nociceptive-specific (NS) neurons (and some wide dynamic range – WDR neurones) in laminae I and V/VI (Marchand, 2008). The spinoreticular tract transmits to neurons having large receptive fields that may cover wide areas of the body and play a role in the memory and affective component of pain (Marchand, 2008).

The spinomesencephalic tract originates from neurons found in almost all dorsal horn laminae (Bhatt, 2015). Their projections reach the periaqueductal grey (PAG), the superior colliculus and the parabrachial nucleus (Bhatt, 2015). This pathway is involved in motor responses to pain and in affective aspects (Bhatt, 2015).

The cervicothalamic tract arises from neurones in the lateral cervical nucleus, and receives inputs from nociceptive neurones from laminae III and IV (Hladnik *et al.*, 2015).

The spinohypothalamic tract originates from NS and WDR neurons in laminae I, V and X (Merighi *et al.*, 2008; Hladnik *et al.*, 2015) that project to the contralateral hypothalamus (Hladnik *et al*, 2015). This pathway is involved in the regulation of the neuroendocrine and cardiovascular responses that accompany pain syndromes (Merighi *et al.*, 2008; Hladnik *et al.*, 2015).

The thalamus is the crucial relay for the reception and processing of nociceptive information in route to the cortex (Fig. 5) (Boadas-Vaello *et al.*, 2016). The thalamo-cortical inputs are mainly conveyed to the first somatosensory area of the postcentral cortical gyrus (S1) (Merighi *et al.*, 2008). However, other cortical areas with nociresponsive or WDR neurons are activated by noxious

stimuli, such as: the second somatosensory area (S2); certain regions of the parietal cortex; the insular cortex; the anterior cingulate cortex; and the medial prefrontal cortex (Merighi *et al.*, 2008). These cortical structures are highly interconnected to each other and with limbic structures and associated with emotional-cognitive factors (Merighi *et al.*, 2008; Ossipov *et al.*, 2010).

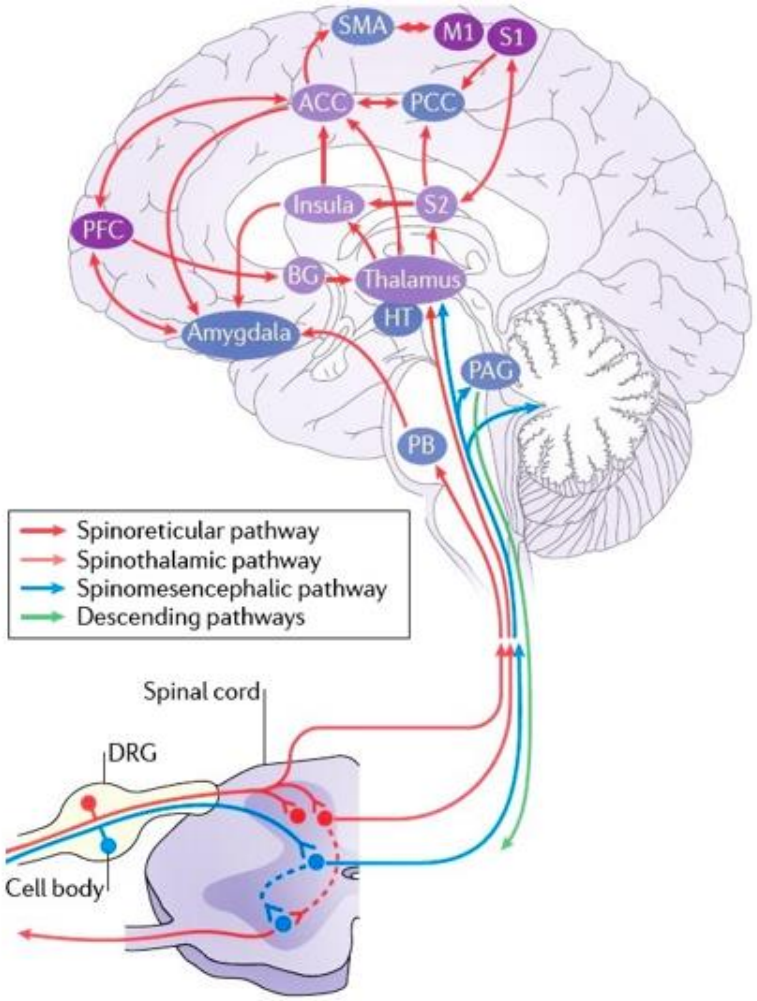

Figure 5. The brain harbours numerous cortical and subcortical structures that are activated via three major ascending pathways, spinoreticular, spinothalamic and spinomesencephalic pathways (shown in red, pink and blue, respectively). The brain markedly modulates spinal nociceptive processing via descending pathways (shown in green). (ACC, anterior cingulate cortex; BG, basal ganglia; HT, hypothalamus; M1, primary motor cortex; PAG, periaqueductal grey; PB, parabrachial nucleus; PCC, posterior cingulate cortex; PFC, prefrontal cortex; S1, primary somatosensory cortex; SMA, supplementary motor area) (Adapted from Kuner & Flor, 2017).

1.6.2 Descending control

As evidenced above, the modulation of pain is not restricted to a single pathway but a multitude of systems, facilitatory and inhibitory, working in parallel (Boadas-Vaello *et al.*, 2016).

The RVM can either facilitate or inhibit nociceptive inputs and acts as a final relay in the control of descending pain facilitation, through which cortical and subcortical sites can influence nociception (Ossipov *et al.*, 2010).

Descending control arises from a number of supra-spinal sites, including the midline PAG-RVM system, the more lateral and caudal dorsal reticular nucleus (DRt) and the caudal ventrolateral medulla (cVLM) (Boadas-Vaello *et al.*, 2016).

Numerous studies over the past half-century have established that activation of midbrain and medullary sites can exert bidirectional control over nociception (Ossipov *et al.*, 2010; Boadas-Vaello *et al.*, 2016). The periaqueductal gray matter (PAG) receives inputs from higher brain centers and is capable of activating a powerful analgesic effect (Ossipov *et al.*, 2010). The rostroventromedial medulla (RVM) neurons receive a dense innervation from the PAG and project to the dorsal horn through the dorsolateral funiculus, forming synapses with spinal cord neurons of the dorsal horn, in both superficial and deep layers (Ossipov *et al.*, 2010; Boadas-Vaello *et al.*, 2016).

DRt stimulation causes hyperalgesia in acute pain, whereas its lesion induces analgesia in both the acute and persistent pains (Boadas-Vaello *et al.*, 2016). The descending pronociceptive fibers from the DRt nucleus establish putatively excitatory synaptic contacts upon lamina I neurons that project back to the DRt (Boadas-Vaello *et al.*, 2016). This nucleus is involved in the maintenance of spinal sensitization in neuropathic pain states (Boadas-Vaello *et al.*, 2016). It also shares reciprocal projections with other brainstem nuclei as the RVM, cVLM, PAG and with forebrain structures like the amygdala (AMY) (Boadas-Vaello *et al.*, 2016).

The cVLM has been shown to display antinociceptive properties, not only by producing profound analgesia after electrical stimulation, but also by tonically inhibiting spinal nociceptive neurons (Boadas-Vaello *et al.*, 2016).

In the spinal cord, pain facilitation comprises the activation of projecting neurones, excitatory interneurones and primary afferents that modulate the activity of NS and WDR neurones, whereas pain inhibition is achieved either by direct inhibition of projecting neurones, desinhibition of relaying inhibitory interneurons or by inhibition of primary afferents (Staud, 2013; Boadas-Vaello *et al.*, 2016).

Growing evidence supports the concept that chronic pain is associated with a dysregulation in descending pain modulation. Disruption of the balance of descending modulatory circuits to favour facilitation may promote and maintain chronic pain (Ossipov *et al.*, 2010).

1.7 Chronic pain

Chronic pain can be divided into (i) nociceptive pain if it results from the activation of secondary nociceptors to the actual damaged area, such as in arthritis, (ii) neurogenic pain, when a cause cannot be identified, but the pain sensation persists due to the abnormal activity of the central nervous system and (iii) neuropathic pain when peripheral or central nervous system injury or dysfunction occurs, such as in sciatic pain (Nicholson, 2006).

A large body of converging evidence suggests that chronic pain is not simply a temporal extension of acute pain but involves distinct mechanisms (Prescott *et al.*, 2014). The transition from acute pain to chronic disorders involves activity-dependent changes (that is, functional plasticity) at many different interconnected levels, ranging from the molecular to the network level, at several anatomical paths in the nociceptive pathway (Sandkühler, 2009; Prescott *et al.*, 2014).

Recent data show that functional plasticity changes are accompanied by structural remodelling and reorganization of synapses, cells and circuits, thereby adding further complexity and a large dynamic range, and potentially accounting for the development of pain that extends over longer periods of time (Fig. 6) (Kuner & Flor, 2017). Structural remodelling of connections has not been studied as widely as functional plasticity, and it remains unclear whether it represents a cause or a consequence of chronic pain (Kuner & Flor, 2017).

Using experimental pain stimulation several studies showed chronic musculoskeletal pain is characterized by more intense and expanded brain activation patterns involving areas, such as somatosensory cortices, the insula (IC) or anterior cingulate cortex (ACC), that tend to correlate with clinical pain duration and by deficient activation of brain circuits that are involved in pain inhibition (Kregel *et al.*, 2015).

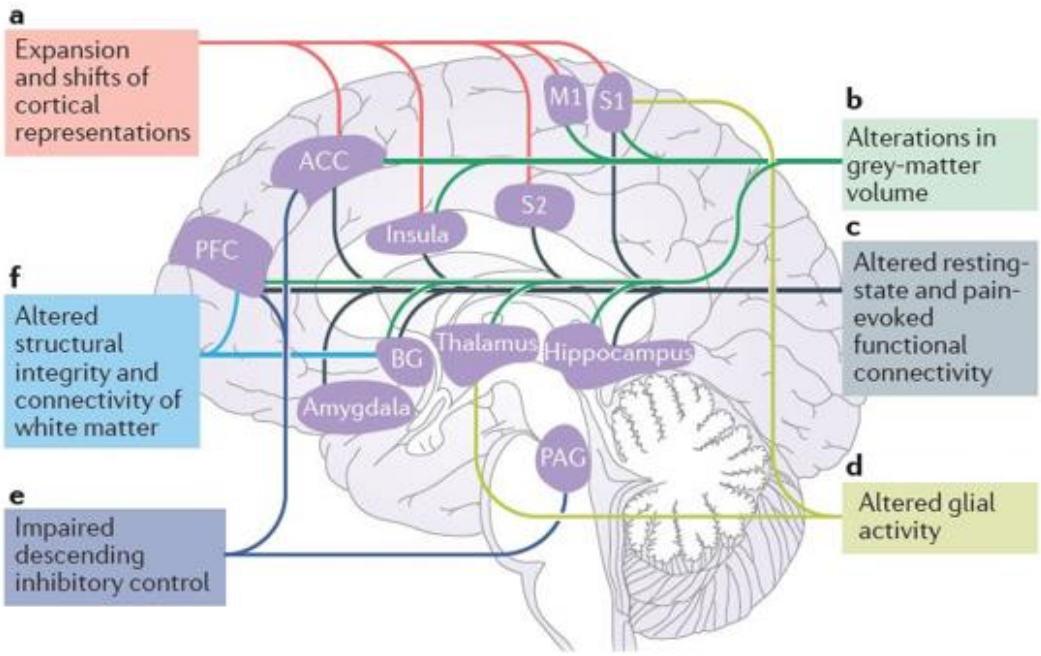

Figure 6. Structural and functional changes in the human brain in chronic pain conditions. a: Brain areas undergoing functional reorganization. b: Regions of grey-matter alterations. c: Altered resting-state and pain-evoked functional connectivity. d: Brain glial activation. e: Changes in activity in descending inhibitory pathways. f: Changes in white-matter integrity and structural connectivity. ACC, anterior cingulate cortex; BG, basal ganglia; M1, primary motor cortex; PAG, periaqueductal grey; PFC, prefrontal cortex; S1, primary somatosensory cortex; S2, secondary somatosensory cortex (Kuner & Flor, 2017).

1.8 Pain and emotional comorbidities

Pain is a highly harmful experience by itself, however, it can also have an overwhelmingly negative effect on almost every other aspect of life, including mood, quality of life and ability to work (Katz, 2002). According to a World Health Organization (WHO) study, people living with persistent pain are four times more likely to suffer from depression or anxiety (Katz, 2002). Concomitant depression and pain disorders are common (Rasmussen *et al.*, 2004; Robinsson *et al.*, 2009). Epidemiological studies report an average prevalence rate (assessed in pain clinics of 52%, and a mean prevalence of pain in depressed patients of 65%) of major depressive disorder (MDD) in patients with chronic pain (Robinsson *et al.*, 2009).

Patients suffering from pain, particularly chronic pain, often display depressive and anxious symptoms (Bair *et al.*, 2008; Thorn, & Kuhajda, 2006). Recent assessments indicate a co-occurrence rate of 30% to 60% of pain and depression (Bair *et al.*, 2003), while other studies (Bair

et al., 2008; Ferreira-Valente *et al.*, 2009; Teh *et al.*, 2010) indicate a prevalence of 18% to 56% of people with chronic pain suffering from a depressive disorder. Other works (McWilliams *et al.*, 2003) found that 35% of individuals with chronic pain displayed anxiety disorders.

Regarding gender differences in anxiety, some studies have shown women tend to report higher levels of anxiety symptomatology compared to men (Fillingim *et al.*, 2009). The same authors report that depression is more prevalent in women with chronic pain and that they present more complaints of pain when compared to men, and pain is differentially perceived between genders (Fillingim *et al.*, 2009; Ferreira-Valente *et al.*, 2009).

Chronic pain may trigger depressive symptomatology and depression, which in turn, increases the adverse effects of pain: depression reinforces chronic pain and chronic pain promotes depressive symptoms, creating a synergistic relationship of negative effect (Teh *et al.*, 2010; Li, 2015).

Anxious and depressive disorders can affect patients diagnosed with OA, since chronic pain caused by the disease increases the risk of these comorbidities (Amorim *et al.*, 2014).

1.8.1 Depression

Depression can be defined as a syndrome or as a disorder (DeRubeis *et al.*, 2008). Individuals suffering from this syndrome suffer from episodes of sadness, loss of interest, pessimism, negative beliefs about themselves, decreased motivation, behavioral passivity, sleep disturbances, appetite and sexual interest, thoughts and suicidal impulses (DeRubeis *et al.*, 2008).

Depression is among the most common psychiatric disorders and presents higher treatment costs (Slavich & Irwin, 2014). This disease can cause great suffering and leads to poor functioning in daily life (Slavich & Irwin, 2014).

It is estimated that one in four women and one in six men suffer from depression during their lifetime, and up to 65% of individuals have recurrent episodes of the disease (Slavich & Irwin, 2014). According to WHO data, depression occurs in 7% of the world's elderly population and accounts for 5.7% of years of disability among individuals over 60 years of age (WHO, 2017). The symptoms of depression in the elderly are often neglected and untreated since they concur with other problems (Slavich & Irwin, 2014, WHO, 2017).

An aggravating factor of this problem is the fact that many people with depression are not diagnosed nor treated, and only about 30% to 35% of treated individuals have reached their normal state through current therapeutic approaches (Slavich & Irwin, 2014).

Depression has been estimated to be the leading non-fatal disease worldwide, so the identification of causes and solutions for the prevention and treatment of depression are of paramount public importance (Slavich & Irwin, 2014).

1.8.2 Anxiety

Generalized Anxiety Disorder (GAD) is defined as excessive apprehension and feeling of bad omen, and its diagnostic frequency ranges from 10% to 45% (Dillon *et al.*, 2013). This disorder is characterized by an excessive and inadequate concern that is persistent and not restricted to particular circumstances (Dillon *et al.*, 2013; Lader, 2015).

Patients display physical symptoms of anxiety (such as tachycardia and tremor) and important psychological symptoms, including restlessness, fatigue, concentrating difficulty, irritability, and sleep disturbances (Lader, 2015). The disorder is common and disabling and the associated functional impairment is similar to that of depression (Lader, 2015). However, many of those who could benefit from treatment are not diagnosed or treated (Lader, 2015).

GAD is the third most common neuropsychiatric symptom of mild cognitive impairment (MCI), and there is some indication that the presence of anxiety in MCI raises the risk of progressing to Alzheimer's disease (Dillon *et al.*, 2013, Gomoll & Kumar, 2015).

Anxiety is a consequence of several underlying and overlapping factors, namely the environment, physical state, underlying brain disease, increased vulnerability due to age and cognitive decline, and psychological/existential problems (Gomoll & Kumar, 2015). Biologically, anxiety is often conceptualized as a complex interaction between various systems within the brain, including the prefrontal cortex, amygdala, the hypothalamic-pituitary-adrenal axis, among other systems involved in emotional processing, conditioned fear, and memory (Gomoll & Kumar, 2015). There is limited evidence for the pathophysiology of increased anxiety in the specific context of neurodegeneration (Gomoll & Kumar, 2015).

1.9 Microglia and pain

Microglia in the healthy mature CNS, including the brain, spinal cord, as well as the eye and optic nerve, have a ramified morphology, a small soma with fine cellular processes. This typical appearance has been associated with microglial "resting" state. Infection, trauma, ischemia, neurodegenerative diseases, or altered neuronal activity, that is any disturbance or loss of brain homeostasis indicating real or potential danger to the CNS can evoke rapid and profound changes in the microglial cell shape, gene expression and the functional behavior which summarily is defined as "microglial activation" or "microgliosis" (Garden & Moller, 2006; Kettenmann *et al.*, 2011; Ginhoux *et al.*, 2013).

The microglial activation process is controlled by exogenous and endogenous 'alarm' molecules ('on' signals) or by suppressed production of microglia-inhibitory molecules that are constitutively produced in the brain, usually by neurons. Central sources of microglial activation include brain-derived pathogen and danger-associated molecular patterns (PAMPs, DAMPs), cytokines, chemokines, inflammatory mediators (ROS, NO) or prostaglandins (PGs), as well as various neurotransmitters, neuromodulators, and hormones associated with these inflammatory challenges (Yirmiya *et al.*, 2015).

There are two main functional aspects of microglia: immune defense and CNS maintenance. As immune cells, they act as sentinels, detecting the first signs of pathogenic invasion or tissue damage. Under the inflammatory conditions of an active immune response however, microglia must also moderate the potential damage to the CNS and support tissue repair and remodeling. Perhaps unsurprisingly, dysregulated microglial activation and microglia induced inflammation is observed in virtually all brain pathologies (such as Alzheimer's disease, prion disease, Parkinson's disease, multiple sclerosis, AIDS dementia and stroke), emerging evidence suggests that microglia exert direct effects on neurons, contributing to disease progression (Tsuda *et al.*, 2005; Ginhoux *et al.*, 2013).

In response to injury, microglia proliferate, migrate to the site of injury and undergo a morphological change to become less ramified and more amoeboid. In terms of function, they can phagocytose cellular debris, present antigens and secrete a broad range of cytokines and chemokines, which amplify the transmission of nociceptive information (Garden & Moller, 2006; Ousman & Kubes, 2012; Thakur *et al.*, 2012).

Recent studies have led to the finding that non-neuronal cells, like glia and immune cells, have a significant role in neuropathic pain development. As experimental animal models and human post-mortem studies of CNS disorders have reliably shown concomitant and co-localized increases in translocator protein (TSPO) expression and markers for activated astrocytes and/or microglia, TSPO expression is widely acknowledged as a marker of glial activation in CNS injury and disease (Loggia *et al.*, 2015).

Chronic pain conditions are also accompanied by significant affective and mood disorders, including depression and anxiety (Taylor *et al.*, 2016).

Microglial activation has been reported in brain regions of patients with major depressive disorder and in preclinical studies has been shown to contribute to the progression and severity of these conditions (Bhattacharya *et al.*, 2016). Although several animal studies have identified chronic pain-induced microglial activation in discrete regions of the brain, including the ventral tegmental area, nucleus accumbens, amygdala, and rostroventral medulla (Dimitroulas *et al.*, 2014; Loggia *et al.*, 2015; Taylor *et al.*, 2016), systematic research to assess simultaneous microglial activation in brain regions involved in sensory and affective dimensions of pain has not yet been conducted.

2.1 Objectives

Several classes of drugs, such as nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are used to treat inflammatory diseases, these have analgesic, anti-inflammatory and antipyretic properties (Gené *et al.*, 2009). However, adverse side effects of NSAIDs and corticosteroids are common, including hypertension, hyperglycemia, muscular weakness, increased susceptibility to infection, osteoporosis, glaucoma, psychiatric disturbances and growth arrest, because of their frequency and severity (Gautam & Jack, 2009; Gené *et al.*, 2009).

For the above mentioned reasons, it is essential to identify new drugs for the prevention and treatment of diseases, including inflammatory diseases. Medicinal plants have been a source of a wide variety of biologically active compounds for many centuries and today challenge is to describe and understand the diversity of active compounds, their action alone or in natural combinations as found in plants (Wink *et al.*, 2015).

The present study aims to evaluate the pharmacological potential of *Baccharis dracuncolifollia* to reverse OA-induced nociceptive and emotional impairment.

More specifically, we aimed to:

1. Characterize the phytochemical profile of Bd extract, regarding their content in phenolic compounds;
2. Evaluate *in vitro* antioxidant properties of Bd extract using several tests, including DPPH, ICA, NO and SO assays and to correlate their scavenging activity with their phenolic compounds in attempt to predict their potential as antioxidants;
3. Evaluate *in vivo* properties of Bd extract: antidepressant, anxiolytic and antinociceptive effects;
4. Evaluate if OA induces neuroinflammation in brain areas involved in somatic and emotive-affective pain modulation, namely in the prefrontal cortex, amygdala and periaqueductal grey matter and if Bd treatment is able to reverse it;

3.1 Plant Material

The plant material used in this study, a hydro alcoholic extract of *Baccharis dracunculifolia* was kindly provided by Professor Silva-Filho, Faculty of Pharmacy and Biochemistry, Pharmacology Department, Federal University of Juiz de Fora, Juiz de Fora, MG – Brazil.

Aerial parts of *Baccharis dracunculifolia* were collected in the garden of the Faculty of Pharmacy of the Federal University of Juiz de Fora (UFJF) in January 2013 (deposited in the Herbarium Leopoldo Krieger (CESJ) of the Institute of Biological Sciences of UFJF, under No. 47.482).

The plant material was dried at room temperature and triturated with a blender. The resulting powder was subjected to a maceration process with hydro alcoholic solution (8:2 v/v) (Ethanol P.A. Vetec) for 3 days. Subsequently, the solution was filtered on filter paper and concentrated with a rotary evaporator (Buchi® RII) under reduced pressure (Buchi® V-700 pump) and bath at 50 ° C.

The extract was lyophilized (Christ® Alpha 2-4, B. Braun) for 2 days and stored in the dark at room temperature.

3.2 High pressure liquid chromatography

To separate, identify and quantify the major phenolic compounds, plant extract was submitted to HPLC analysis. HPLC analysis was carried out in a Hitachi-Merck HPLC-DAD system (high performance liquid chromatography with diode array detection), controlled by Merck pc- software (Merck, New York, USA). The chromatrograph 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. The injected volume was optimized to 20 µl of Bd extract (concentration 3mg/ml) and was injected to perform the separation of compounds.

The solvent system used was a gradient of methanol P.A. (Merck, Darmstadt, Germany) with 0.1% of formic acid (Merck, Germany) (solvent A) and water with 0.1% of formic acid (Merck, Germany) (solvent B). The optimal elution profile consisted of a six-step gradient: starting with 15% of solvent A and installing a gradient to obtain 15% A at 3 min, 90% (solvent A) at 35 min, 90% (solvent A) at 45 min, 15% (solvent A) at 50 min and 15% (solvent A) at 60 min.

Detection was performed with a diode array detector. Spectral data from all peaks were accumulated in the wavelength range 245-530 nm and chromatograms were recorded at 260, 280 and 350 nm.

The phenolic compounds present in the samples were characterized according to their UV-Visible spectra and retention times compared with commercial standards reference compounds, according to protocol of Dias *et al.* (1999).

3.3 Evaluation of antioxidant activity

Studies of antioxidant activity in plants attempt to relate the antioxidant activity with its phenolic content and the properties of other plant compounds of high interest (Dudonné *et al.*, 2009). The evaluation of the properties of the compounds is a common procedure and extremely necessary for chemical and pharmacological studies (Keating *et al.*, 2014), with the effects of antioxidants already being recognized as cellular defenses against oxidative processes (Dudonné *et al.*, 2009).

3.3.1 DPPH assay

The antioxidant activity of the extract was determined by the free radical sequestration method DPPH (2,2-diphenyl-1-picryl-hydrazyl) as described by Silva *et al.* (2008). To evaluate the antioxidant activity using this method, a series of dilutions were prepared from the stock solution (150 mg / mL in DMSO): 10 µl extract (100% ethanol) and 140 µl DPPH (stock solution, 400 µM, ethanol 100%, Sigma) for a final concentration range of 1 mg / ml to 0.01563 mg / ml.

For each concentration three independent replicates were performed and the absorbance was monitored over 60 minutes at 515 nm with a microplate reader (SpectraMax Plus 384, USA). The blank of the samples and the negative control consisted of 10 µl of the respective solutions at each concentration and 140 µl of 100% ethanol and 140µl DPPH, respectively. The DPPH solution was pre-prepared and stored at 4° C in the dark between analysis.

After 60 minutes of reaction, the ability of the samples to reduce 2,2-diphenyl-1-picryl hydrazyl to 2,2-diphenyl-1-picryl hydrazine was evaluated by changing the color of samples the purple to yellow, detected by decreasing the absorbance.

From the absorbance readings, the percentage of antioxidant activity (% AA) corresponding to the amount of DPPH reduced by the samples was determined using the following formula: % AA = ((Abs C - (Abs t60 - Abs B)) / Abs C)) * 100, where Abs t60: absorbance of the samples for each

concentration in time 60 minutes; Abs B: absorbance of the blank of the samples for each concentration at time 60; Abs C: absorbance of negative control.

The efficient concentration (EC) was obtained from the percent reduction of the DPPH curve of each extract necessary for a 50% discoloration. The higher DPPH reduction by the sample, the lower the 50% efficient concentration (EC50) and means higher antioxidant activity.

3.3.2 ICA assay

The iron chelating activity of the extract was determined by the protocol described by Russo *et al.*, 2005. In this test, a series of dilutions were used: 50µl of extract (100% ethanol), 50µl of stock solution of FeSO 4 (0.12mM, (0.6 mM ultrapure water), for a final concentration range of 3 mg / ml to 0.09375 mg / ml. Three independent replicates were performed for each concentration.

The absorbance reading was performed after 10 minutes incubation in the dark at room temperature at 515 nm with a microplate reader (SpectraMax Plus 384, USA). The blank of the samples and the negative control consisted of 50 µl of the respective solutions at each concentration and 50 µl of water, respectively, plus 50µl of FeSO4 stock solution (0.12 mM, ultrapure water) and 50µl of stock solution of Ferrozine (0.6 mM ultrapure water). Stock solutions of FeSO4 and ferrozine were pre-prepared and stored at 4 ° C in the dark between analyzes.

The ability of the samples to inhibit the formation of the Fe-ferrozine complex was observed by changing the color of sample from purple to yellow, detected by decreasing the absorbance.

From the absorbance readings, the percentage of iron chelation corresponding to the amount of inhibition of Fe-ferrozin complex formation was determined using the following formula: %ICA= (((Abs C - (Abs – Abs B)) / Abs C)) x 100, where, Abs: absorbance of samples for each concentration; Abs B: white absorbance of samples for each concentration; Abs C: absorbance of negative control.

After calculating the percentages of the iron chelation capacity of the samples, the values obtained were used to perform the graphs, whose abscissa corresponds to the concentration of the sample and the ordinate to the% ICA. The EC 50 was obtained from the percentage of each extract required for a 50% discoloration.

3.3.3 NO assay

In order to evaluate the nitric oxide inhibition capacity of the extract, a protocol by Colle *et al.*, 2012 was applied. The dilutions used were: 50 µl of extract (phosphate buffer pH 7.4), 50 µl of stock solution of SNP (20 mM, ultrapure water) and incubated for one hour at a succession of concentrations between 1 mg / ml and 0.0315 mg / ml. Three independent replicates were performed for each concentration.

After the suggested incubation time, 50 µl of Griess reagent (excluding the sample and control blank) was added. The Griess reagent was formed by 50% 1% sulfanilamide in 5% H 3 PO 4 and 50% NED (0.1%, ultrapure water).

Absorbance reading was performed after 10 minutes incubation in the dark at room temperature at 560 nm with a microplate reader (SpectraMax Plus 384, USA). The blank of the samples consisted of 50 µl of extract (phosphate buffer pH 7.4) and 50 µl of stock solution of SNP (20 mM, ultrapure water), and the control blank and the negative control were 50 µl phosphate buffer pH 7.4 and Of 50µl SNP (20mM, ultrapure water).

Stock solutions of sulfanilamide (1% in 5% H3PO4) and NED (0.1%, ultrapure water) were pre-prepared and stored in the dark between analyzes, however Griess reagent was produced again at each independent replicate. The ability of the samples to inhibit the formation of the nitric oxide was observed by changing the color of the sample from pink to yellow.

The percent inhibition of nitric oxide was determined by the following formula: % inhibition of NO = ((((Abs C-AbsBC) - (AbsBA – Abs BA)) / (AbsC-AbsBC))*100) Absorbance of the samples for each concentration Abs BA: white absorbance of the samples for each concentration Abs C: control absorbance Abs BC: control white absorbance.

After calculating the percentages of the nitric oxide inhibition capacity of the samples, the values obtained were used to perform the graphs, abscissa corresponds to the concentration of the sample and the ordinate to% NO inhibition. The EC 50 was obtained from the percentage of each extract required for a 50% discoloration.

3.3.4 SO assay

Superoxide production by the extract was evaluated according to the protocol reported by Valentão *et al.*, 2002. The range of dilutions used were: 50µl of extract (phosphate buffer pH7.4), 50µl of

stock solution of NADH (1.97mM in phosphate buffer PH 7.4) 150 µl NBT stock solution (81.5 µM in phosphate buffer pH 7.4) and 50 µl PMS solution (3.26 mM in phosphate buffer pH 7.4). The final range of concentrations used was between 1mg / ml and 0.03125 mg / ml. Three independent replicates were performed for each concentration.

Absorbance reading was performed after 2 minutes of incubation at room temperature, at 560 nm with a microplate reader (SpectraMax Plus 384, USA). The blank of the samples was composed of 50 µl of respective solutions at each concentration and 50 µl of NADH stock solution (1.97 mM in phosphate buffer pH 7.4) 150 µl of NBT stock solution (81.5µM in phosphate buffer pH 7.4) and Negative control by 50 µl phosphate buffer pH7.4, 50 µl NADH stock solution (1.97 mM phosphate buffer pH 7.4) 150 µl NBT stock solution (81.5 µM in phosphate buffer pH 7.4) and 50 µl PMS solution (3.26 MM in phosphate buffer pH 7.4). The reaction starts with the addition of PMS (3.26 mM in phosphate buffer pH 7.4).

The stock solutions were pre-prepared and stored in the dark between analyzes.

The ability of superoxide production samples was observed by changing the color of the sample from blue to yellow detected by the decrease of absorbance.

The percentage of superoxide production was determined using the following formula: % SO = (((Abs C - (Abs - Abs B)) / Abs C)) * 100, where Abs: absorbance of samples for each concentration; Abs B: white absorbance of samples for each concentration; Abs C: absorbance of negative control.

After calculating the percentages of the superoxide production capacity of the sample, the values obtained were used to perform the graphs, whose abscissa corresponds to the concentration of the sample and the ordinate to the% SO. The EC 50 was obtained from the percentage of each extract required for a 50% discoloration.

3.4 In vivo assay of the pharmacological potential of *B. dracunculifolia*

3.4.1 Ethical considerations and handling

The experimental protocol was approved by the Institutional Ethical Commission and followed the European Community Council Directive 2010/63/EU concerning the use of animals for scientific purposes. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

The animals were kept in a controlled temperature environment, 22°C, 55% relative humidity, 12h light / dark cycle (dark phase starting at 8 pm), in standard polycarbonate cages (45.4x 25.5x 20 cm) with a maximum of three animals per cage and with access to water and food (F0021; BioServ, Flemington, USA) *ad libidum.*

The health general parameters were taken into consideration and evaluated weekly by the resident veterinarian throughout the experimental period. Additionally, animals were weighted weekly.

Before start the experiment, all animals were handled daily by the researched for 15 days. On the day of the experimental sessions animals were left in the experimental room for an hour in order to habituate to the surroundings and the protocols were conducted during the light phase of the cycle, except the sucrose preference test (SPT) that was performed during the dark cycle. All efforts were made to ensure that the experimenters were blind to the treatment.

3.4.2 Anesthesia and euthanasia

For the ovariectomy and the induction of experimental OA, the animals were anesthetized with a mixture of ketamine (0.75 mg/Kg animal, Imalgene, Merial, Lyon, France) and medetomidine (0.5 mg/Kg animal, Dorbene, Esteve Veterinaria, Léon, Spain) injected intraperitoneally (ip) (Syringe: 0.6x25mm, Terumo, Neolus, Belgium) (David-Pereira *et al.*, 2016). A sufficient depth of anesthesia was frequently monitored by observing the size of the pupils, the general muscle tone and was judged from the absence of nociceptive withdrawal reflexes. A blanket was used to maintain the body temperature within physiological range

After surgery, anesthesia was reversed by administration of atipamezole hydrochloride (1 mg / kg animal, i.p., Antisedan, Orion Pharma, Orion Corporation, Espoo, Finland). The animals were monitored until fully recovered (grooming and eating).

After the end of the experimental period, the animals received a lethal dose of sodium pentobarbital.

3.4.3 Ovariectomy

Ovariectomy was performed to mimic postmenopause in women. The ovariectomy followed a protocol as described by Lasota & Klonowska (2004). Before performing the ovariectomy, the animals were placed in ventral recumbency and shaved to remove the hair over their lumbar spine

(Sophocleous & Idris, 2014). The shaved area was cleaned with braunol (B. Braun, Melsungen AG, Tuttligen, Germany) to disinfect the skin.

Using sterile small scissors, a longitudinally dorsal incision of 1–2 cm was made along the lumbar vertebrae (approximately half way between the middle of the back and the base of the tail), and the skin on each side of the cut was separated from the underlying muscle using blunt end forceps. The same skin incision was used to remove both ovaries, because the skin in rodents is so loose that it can be retracted from side to side (Fig. 7). Ovaries were easily located because they were embedded in the fat pad, which is visible underneath the muscle (Sophocleous & Idris, 2014).

While holding the muscle layer with sterile serrated forceps, the ovary together with the associated ovarian fat pad was gently withdrawn using sterile blunt forceps. This allows the ovary, oviduct and part of the uterus to be exposed. While taking care not to disturb the ovary, a suture was tied and knotted tightly around the cranial portion of the uterus and uterine vessels, about 1 cm distal to the ovary. This was performed to prevent excessive hemorrhages following the removal of the ovary (Sophocleous & Idris, 2014). The exposed ovary, oviduct and part of the uterus were carefully removed using sterile scissors, whereas the caudal portion of the uterus was replaced in the cavity.

The muscle layer and the skin were sutured with silk thread (Muskle layer: 3/0 Mersilk (W571H); Skin: 2/0 Monocryl (W3448), Ethicon, B. Braun, Tuttligen, Germany) and the wound cleansed with braunol (B. Braun, Tuttligen, Germany). This procedure was performed in all animals.

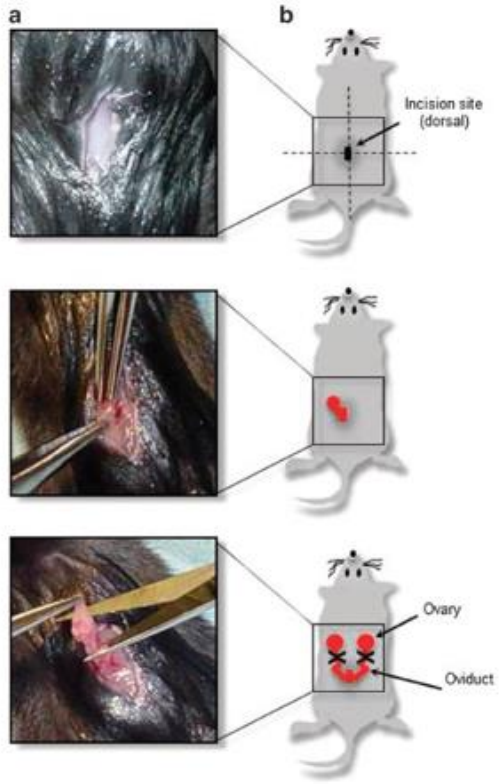

Figure 7. A visual demonstration (a) and schematic illustration (b) of the ovariectomy procedure using a single dorsal midline skin incision (adapted from Sophocleous & Idris, 2014).

At the end of procedure, animals were returned to their home cage and were monitored for signs of pain or distress over the following 72h.

3.4.4 OA induction

The induction of OA was performed as described by Amorim *et al.* (2014). A mixture of 0.1 ml of 3% carrageenan 3% kaolin (Sigma-Aldrich, St Louis, MO, USA) dissolved in a sterile saline solution (0.9% NaCl pH 7.2, Unither, Amiens, France) was injected into the right knee joint with a hypodermic syringe (0.3x13 mm, Microlance, UK). This model induces mechanical hyperalgesia, which starts within a few hours after surgery and lasts several weeks (Radhakrishnan *et al.*, 2003; Amorim *et al.*, 2014). The animals of the SHAM group were injected with 0.1 ml of saline solution (0.9% NaCl pH 7,2, Unither, Amiens, France) into the right knee synovial cavity. To guarantee a mixture distribution and to initiate the degradation of the cartilage, the paw of the animal was flexed and extended ten times. At the end of the induction session all boxes were returned to the animal house.

3.4.5 Drug preparation and administration

Powdered extract of Bd was dissolved in PBS 10mM (137mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, Tween 20 1% (Applichem, Panreac, ITW companies, Barcelona, Spain), pH 7.4) for a final concentration of 50 mg/ml of Bd, placed on an ultrasound bath (Sonicator Branson 2510) for 30 minutes and stored in the dark at room temperature.

The concentration of the extract administered to each animal was adjusted weekly according to the animals' body weight so that the final volumes administered (1 ml for Bd50 group and 1.5 ml for Bd100 group) contained 50 mg Bd/kg and 100 mg Bd/kg, respectively (Dos Santos *et al.*, 2010; Rezende *et al.*, 2014). Prior to administration, the extract solution was placed on the ultrasound device for 15 minutes to dissolve the extract completely.

Administration of the drug was performed daily for 5 weeks through gavage with the aid of an orogastric cannulae. SHAM and ARTH animals were administered the vehicle solution. In the week preceding the start of the Bd treatment, all animals were trained daily for the gavage procedure.

3.5 Behavioural analysis

3.5.1 Pressure application measurement (PAM)

The application of noxious pressure to the primary site of injury is a classical approach to measure mechanical hyperalgesia, both in humans and animals (Amorim *et al.*, 2014). In our work, this evaluation was performed using the pressure application measurement (PAM) method (Amorim *et al.*, 2014). This method allows an accurate behavioural measurement of primary mechanical hypersensitivity in rodents with chronic inflammatory joint pain by the application of a force range of 0 – 1500 g directly to the affected joint (Amorim *et al.*, 2014).

The PAM apparatus (Ugo Basile, Comerio, Italy) is composed of a force meter coupled to a sensor that is used on the operator's thumb. With the animal securely held and an increasingly force was gradually applied across the joint of interest (the experimenter's thumb on one side of the animal's knee joint and the index finger on the other side) until it reaches the maximum force required to elicit a response: paw withdrawal, vocalization or wriggling movements. The results correspond to the force peak applied immediately before the response and are presented in grams force (gf) (Barton *et al.*, 2007; Malfait *et al.*, 2013).

The test was performed twice (i) before the start and at (ii) the end of pharmacological treatment and measurements were recorded as the limb withdrawal threshold (LWT). LWT was measured twice on the knee joints in each limb (right hind paw and left hind paw) at 1 min intervals. The mean LWTs were calculated per animal. At the end of the session animals were returned to their home cage.

Analysis of data from the PAM test were performed using the difference between the PAM results of each animal obtained prior to BD administration and PAM results after Bd treatment, in order to avoid bias related to individual differences between animals in term of mechanical sensitivity. So, PAM = (LWT prior to Bd treatment – LWT after Bd treatment).

3.5.2 Open field test (OFT)

The open field test (OFT) was used to evaluate differences in the anxiety-like behaviour between animals and followed a protocol previously described by Amorim *et al.* (2014).

The OF apparatus consists in a square arena with 100 cm (W) x 100 cm (L) x 40 cm (H). The test room was dimly lit, with a gradient of light from the center to the periphery. A single rat was placed in the center of the floor and its behaviour recorded with the help of a video camera located 40 cm above the arena. The test started when the animal was placed at the center of the arena and its exploratory activity was automatically registered during 5 min.

Anxiety-like behaviour was evaluated through the analysis of the following parameters: (1) number of squares crossed (using the front paws and ears as reference); (2) the number of rearings (vertical activity) and (3) the time spent in the center versus time spent at the periphery. After each test, the area was cleaned with 90% alcohol solution to removed environmental odors (Mesquita *et al.*, 2006; Saenz *et al.*, 2006; Gonçalves *et al.*, 2008). At the end of the session animals were returned to their home cage.

The time in the central and peripheral areas were used to calculate the proportion of time spent in the central quadrant of the platform. The downtime, rearings and number of squares crossed was also registered and counted. The evaluation was performed by two separate researchers blind to the experimental group and treatment group.

3.5.3 Forced swimming test (FST)

Learned helplessness evaluated in the modified forced-swimming test (FST) was used as an index of depressive-like behavior and followed a protocol described previously by Rénéric *et al.* (2002).

The FST is a two-day procedure in which rats are forced to swim under conditions where escape is not possible. On the first day, animals were submitted to a pre-test session (10 min) in which they were individually placed in a clear pexiglass cylinder 50 cm (H) x 29 cm (W) filled with water (21.5 ± 1.5 cm, 24 ± 0.5 °C). After 10 minutes the rats were removed from the water, dried with towels and placed in their home cage. Twenty-four hours later animals were again placed in the cylinders for a period of 5 min and the testing session was recorded using a video camera.

In the first day, animals learn they cannot escape the cylinder - learned helplessness - and in the second day a decline in the latency to immobility and an increase in the time spent immobile are associated to learned helplessness (Nam *et al.*, 2014).

Behavior measures are defined as follows: (1) immobility: lack of movement of the whole body, except for small movements required to keep the animal's head above water level; (2) struggling: vigorous movements with the front paws in and out of the water, usually directed against the cylinder wall; (3) swimming: making active swimming motions, more than those necessary to avoid drowning, i.e. moving around in the cylinder; (4) latency to immobility: corresponds to the time the animal stop for the first time after the beginning of the test (Rénéric *et al.*, 2002; Amorim *et al.*, 2014).

Test sessions were scored by two researchers blind to the experimental group and the treatment group. Scores were expressed as total behavioural counts per 5min session. Learned helplessness behaviour was defined as an increase in time of immobility at the expense of the time spent swimming/struggling and a decrease in the latency to immobility (Amorim *et al.*, 2014; Amorim *et al.*, 2017).

3.5.4 Sucrose preference test (SPT)

Anhedonia, measured as a reduction in sucrose preference (SPT), was assessed through the application of a protocol adapted from Bessa *et al.* (2009)

This protocol includes an initial exposure of the animals to the sucrose solution (Labchem, Laborspirit, Portugal) for 2 hours, in order to certify that all animals tried the sucrose solution. After,

during the test session, performed during their active period (8:00 p.m.), animals had free access to two bottles, one filled with water and another with the sucrose solution for 12h solution. At the start of the test, all animals were housed for 12 hours in individual cages (see housing conditions). Two pre-weighted bottles were available in each cage, one with 3% sucrose solution and the other with distilled water. At the end of 12 hours, the bottles were removed and weighted. Consumption was noted and the animals were returned to their previous housing conditions.

Sucrose preference (SP) was determined by the evaluation of sucrose consumption (ml), water consumption (ml); and total liquid consumption using the equation:

$$\text{Sucrose\_preference} = \left[\frac{\text{sucrose\_intake}}{(\text{sucrose} + \text{water})\text{\_intake}}\right] \times 100.$$

The SP results were then adjusted to the body weight of each animal.

Anhedonia was defined as a reduction in sucrose preference relative to baseline levels (Bessa *et al.*, 2009).

3.6 Sacrifice

At the end of experimental procedures, the animals were sacrificed with a lethal dose of sodium pentobarbital (200 mg/Kg animal, i.p., eutasil, OrionPharma, Espoo, Finland) and the internal organs (thymus, lung, heart, spleen, liver, kidneys and adrenals) were sampled and the brain excised from each animal.

After weighting the internal organs on a precision scale (Sartorius, Germany), a gross examination was performed, and, they were subsequently stored in 4% PFA (DAC, Applichem, Panreac, ITW companies, Barcelona, Spain) until further processing.

The brain was divided into right and left hemispheres and the right hemisphere was stored in 4% PFA for observation and quantification of activated microglia.

3.7 Histological processing and analysis of the internal organs

Tissues for microscopic examination were stained with haematoxylin and eosin, as described by Oliveira *et al.* (2010).

Firstly, the samples were dehydrated in an increasing gradient of ethanol/water solution (from 90 to 100%) using an automatic slide stainer (Leica AutoStainer XL, USA) for 19 h followed by washing thrice with xylene. Then, samples were immersed in parafin (Thermo Scientific, UK) at 62 °C and allowed to solidify at −5 °C. Slides were prepared by cutting the tissue into 4 μm thick sections (kidney and lungs: Coronal plane; thymus, heart, spleen, liver, adrenals, stomach and esophagus: transverse plan) and mounted in a micro-slide glass (25x75x1mm, Superfrost Plus, Thermo Scientific, UK). Paraffin was melted by placing the slides in an oven at 71 °C for 20 min and allowed to cool down at a room temperature. The remnant paraffin was then eliminated by submersion in 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. Before, slides were immersed thrice in 100% ethanol for 2 min each time of immersion.

For the Haematoxylin and Eosin staining, slides were transferred to a xylene solution (C_8H_{10}, Carlo Erba, Val de Reuil, Cedex, France) and washed twice (one time for 3 minutes and other for 2 minutes), washed again twice with absolute ethanol (one time for 2 minutes and other for 1 minutes) and transferred to a 96% ethanol solution (1 minute) and washed with tap water (1 minute). The staining step consisted in the immersion of the slides into the haematoxylin Harris (Millipore Corporation, USA) for 1 min followed by a bath in tap water for 3 and a half minutes. Then, slides were washed with 0.5% of ammonia solution (Sigma Aldrich, Spain) for 10 seconds, washed with tap water for 1 and half minutes and washed with 96% ethanol solution (45 seconds). Slides were then immersed in eosin Y solution (Thermo scientific, Cheshire, UK) for 30 seconds and dehydrated with 96% ethanol (1 minute), absolute ethanol (2 minutes each time) and xylene (first wash for 1 minute and the second for 2 minutes). Finally, they were immersed thrice in an ethylene/propylene mixture and carefully mounted, to avoid the formation of air bubbles, for observation.

Histopathological examination of tissues was performed in order to screen for potential abnormalities cause by the Bd treatment as compared to the control group. All slides were examined under a light microscope (Olympus BX61, Olympus Co. Ltd, Japan).

3.8 Microglia Staining

3.8.1 Brain processing

After sacrifice, the brains were excised, preserved in 4% PFA for a week and placed in an 20% sucrose solution for 48 h. They were then sectioned in a vibratome (Leica, Carnaxide, Portugal) in coronal sections 50 μm thick and stored in 12-well plates with 0.1 M PBS at 4 °C until further processing as described by Amorim *et al.*, (2014b).

The brain sections were serially collected in 3 sets and one set was used to evaluate microglial activation through IBA-I immunohistochemistry.

3.8.2 Immunohistochemistry for IBA-I

IBA-I immunohistochemistry was performed in brain sections representative of the whole brain; the protocol was adapted from Amorim *et al.* (2014b).

Brain sections were first washed thrice with PBS 0.1M, prepared from a PBS (1M) stock solution (PBS (1M) stock solution was prepared with 160 g of NaCl, 4 g of KCl, 28.8 g of Na2HPO4; Sigma Aldrich, Sintra, Portugal). Sections were then incubated for 30 minutes in 3.3% hydrogen peroxide solution (H_2O_2, Carlo Erba, Val de Reuil, Cedex, Dasit Group, France) prepared in PBS 0.1M. After incubation, sections were washed once with PBS 0.1M and twice with PBS/T 0.3% (prepared with Triton-X 100; Sigma-Aldrich, Sintra, Portugal; PBS 0.1M, pH=7.2). Brain sections were then incubated for 2 hours in 2.5% fetal bovine serum (FBS; Biochrom, Cambridge, United Kingdom), followed by overnight incubation with the primary antibody IBA-I (Abcam, Cambridge, UK) (1 μL antibody: 1000 μL PBS/T + FBS 2%), at room temperature.

The following day, sections were washed in PBS/T (thrice), followed by incubation for 1 hour in biotinylated swine anti-rabbit secondary antibody (1 μL: 200 μL PBS/T; Dako, Denmark). Sections were washed in PBS/T (thrice), followed by incubation with avidin biotin complex solution (ABC, prepared in PBS/T 1 μL: 200 μL; Vectastain, Vector Laboratories, Peterborough, USA) for 1 hour. Then, the sections were washed consecutively with PBS/T (thrice), PBS (thrice) and thrice in Tris buffer (prepared with trizma base 0.05M, Ultrol Gade, Calbiochem, USA and distilled water). Finally, the sections were stained for 2-5 minutes with a diaminobenzidine solution (DAB; Sigma Aldrich) dissolved in Tris buffer (20 mg DAB+40 mL Tris+8 μL H_2O_2). At the end of the reaction, the sections were washed with Tris (twice) and PBS (twice).

After the immunohistochemistry protocol, sections were mounted in microscope glass slides (25x75x1mm, Superfrost Plus, Thermo Scientific, UK) and allowed to dry for one week.

Subsequently the sections were dehydrated in increasing concentrations of ethanol (20%, 40%, 70%, 90% and absolute ethanol in consecutive washes for five minutes, immersed in xylene (C_8H_{10}, Carlo Erba) for five minutes and mounted with Entellan (Merck, Germany).

Microglial activation was quantified separately in each area of the prefrontal cortex, namely in the cingulate (CC), prelimbic (PLC) and infralimbic (ILC) cortices (Fig. 8A), in the amygdala (Fig. 8B) and in and in the dorsolateral (DlPAG) and lateral (LPAG) areas of the periaqueductal grey matter (PAG) (Fig. 8C).

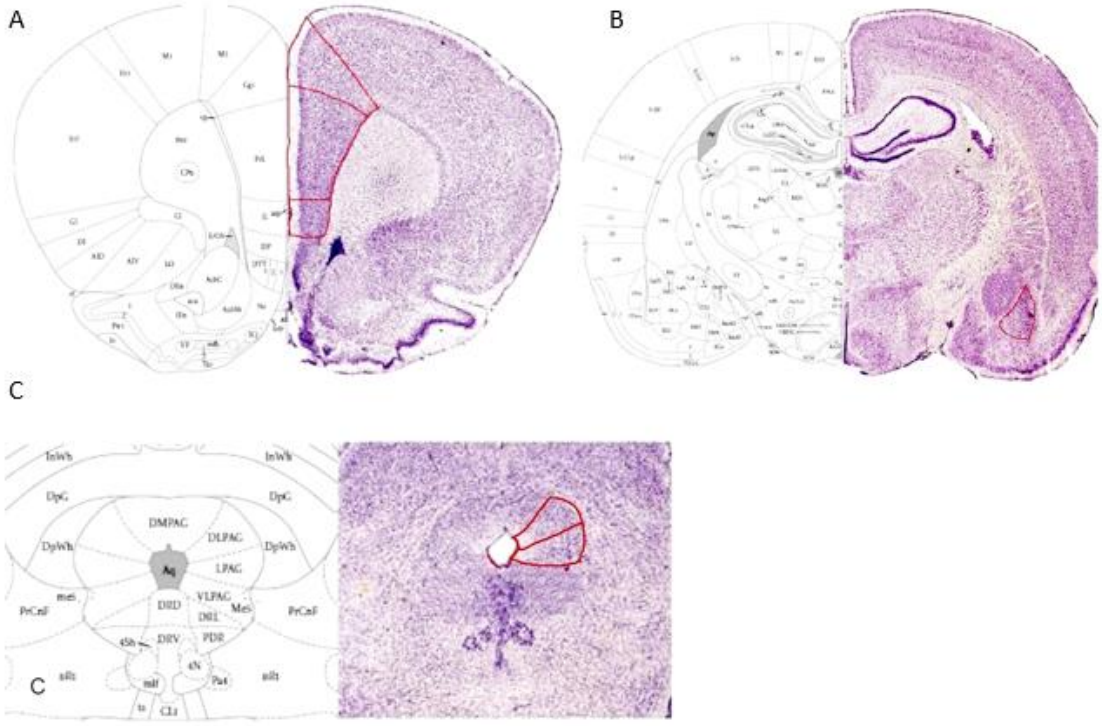

Figure 8. Schematic representation of the prefrontal cortex (PFC)(A), amygdala (AMYG)(B), and periaqueductal grey matter (PAG)(C). Adapted from Paxinos & Watson (2005).

A minimum of 3 sections per animal encompassing the abovementioned brain regions were randomly sampled from each rat and analyzed.

Microglia cells quantification was performed through the optical fractionator method using the Stereo Investigator 10 software (Microbrigthfield Bioscience, Madgedurg, Germany) and a video camera (Sony, Color Video camera, 3CCD, Exwavw HAD) coupled to a microscope (Axioplan2 Imaging, Zeiss, Olympus Co Ltd, Japan). By using the optical fractionator system, a grid (100X100

μm) was overlapped in the target brain areas to analyses in order to have the representative result for that region.

Brain sections were also analyzed for the presence of microglia in different stages of activation divided in moderately (Fig. 9, stage 2 to 4) and highly (Fig. 9, stage 5 to 6) activated (Kreutzberg, 1996).

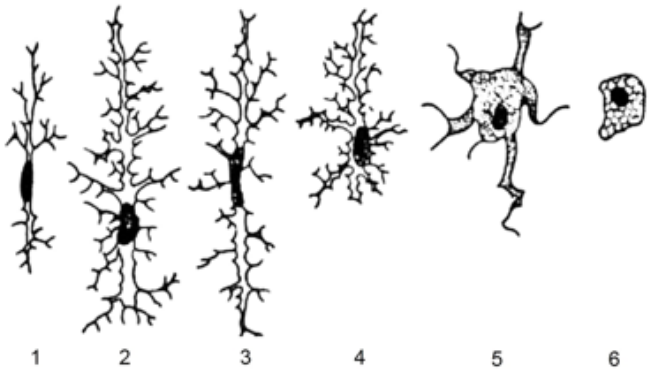

Figure 9. Morphological changes occurring during the progressive activation of microglia. From inactivate state (1) to the most activated form of microglia (6). (Kreutzberg, 1996).

3.9 Experimental design

Upon arrival animals were randomly distributed three by cage and were quarantined. One week later the animals were transfered to the housing facility and daily handling was performed for 15 min during 2 weeks.

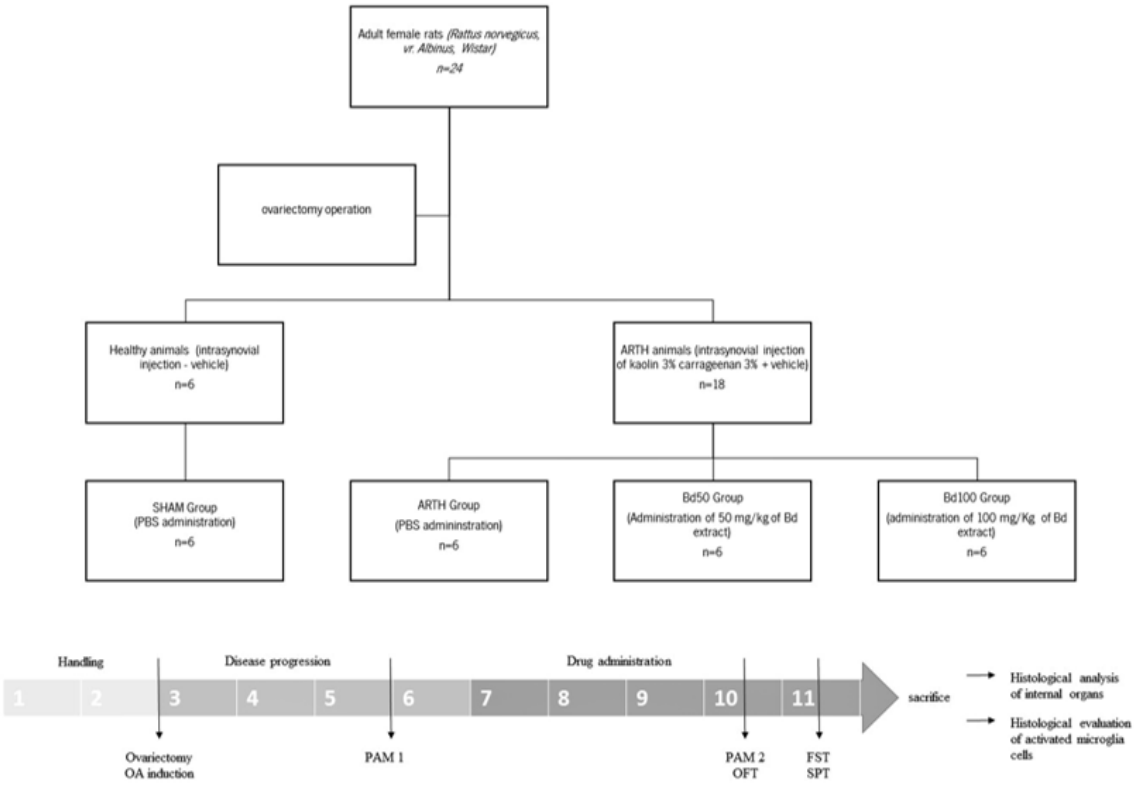

Figure 10. Schematic representation of the experimental design and time points. Animals were divided into 4 groups: (n = 6, each), one control group (SHAM) and the other 3 groups with experimental arthritis: one group without treatment (ARTH); one group with treatment of 50mg/Kg (Bd50) extract and one group with treatment of 100mg/Kg (Bd100) extract. Ovariectomy and OA induction were performed 2 weeks after the beginning of experimental period. Mechanical hyperalgesia was assessed using the PAM one week prior to drug administration and 4 weeks later. Anxious-like behaviour was evaluated using the OFT on the 4th week of treatment, depressive-like behaviour was evaluated using the FST and SPT on the 5th treatment week. At the end of experimental period animals were sacrificed, the internal organs and the brain excised for histopathological analysis and quantification of activated microglia cells.

Two weeks after habituation to the researcher, the apparatus and the experimental room, all the animals underwent the ovariectomy surgery.

Under the same anesthesia, animals included in the ARTH, ARTH+Bd50 and ARTH+Bd100 groups underwent the induction of experimental OA. After the surgery, the anesthesia was reversed, the animals were monitored until fully recovered and returned to their home cages.

Four weeks after OA induction, the administration of Bd extracts was initiated and animals received daily treatment through gavage for a period of 5 weeks. In the last week of treatment, mechanical hyperalgesia (PAM) was evaluated as well as anxiety- (OFT), and depressive-like (FST and SPT) behaviour. At the end of the behavioural sessions animals were sacrificed with a lethal dose of pentobarbital and the internal organs and the brain were removed for further processing and analysis (Fig. 10).

3.10 Statistics /DATA analysis

Statistical analysis was performed using the GraphPad Prism 5 software (GraphPad Software Inc, La Jolla, CA, USA). T-tests were used to evaluate the antioxidant capacity of the Bd extract. For the analysis of the behavioral data and quantification of activated microglia a one-way analysis of variance (ANOVAone-way) followed by *post-hoc* Bonferroni's test for comparisons between groups was used.

$P < 0.05$ was considered to represent a significant difference. Results are expressed as mean ± standard error (SEM).

4.1 Phytochemical profile of *B. dracuncufolia*

The Bd extract is composed by a mixture of phenolic compounds, mostly by caffeoylquinic acids (caffeoylquinic acids concentration: 237,27ug/mg extract), as shown in the HPLC chromatogram (Fig. 11). The peaks of the compounds are presented in figure 11 and their respective concentration is presented in table 1.

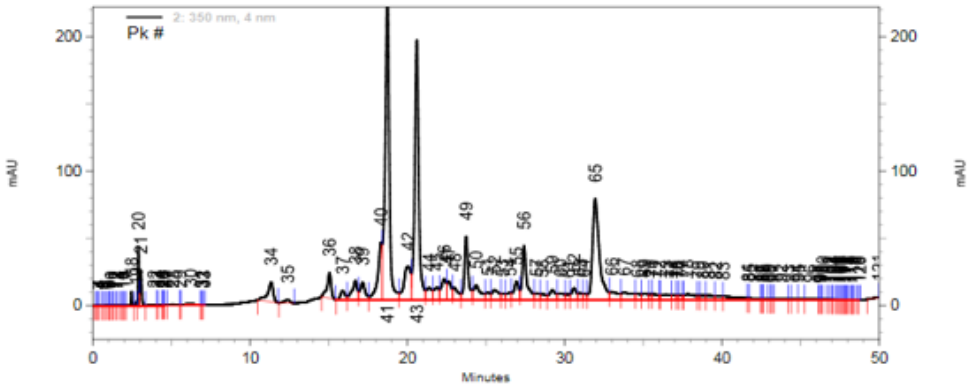

Figure 11. HPLC–DAD-UV (350 nm) phenolic profile of the hydroethanolic extract from *Baccharis dracunculifolia* (Peaks 34, 37, 38, 41, 42, 43, 46, 49 – Caffeoylquinic acid; Peak 36 – Apigenin).

Table 1. Phenolic composition and total phenolic content of the Bd extract (flavanones expressed in equivalents of luteolin-7-O-glucoside and caffeoylquinic acid expressed in equivalents of chlorogenic acid).

Peak	Rt (min)	Proposed compound	Area	Concentration (µg/ml)	Extract concentration (µg/mg)
34	11,33	Caffeoylquinic Acid	1458345	24,745	8,248
36	15,05	Apigenin	1360477	4,513	1,504
37	15,88	Caffeoylquinic Acid	558928	9,484	3,161
38	16,69	Caffeoylquinic Acid	1335437	22,659	7,553
41	18,73	Caffeoylquinic Acid	17256071	292,797	97,599
42	20,05	Caffeoylquinic Acid	3031484	51,438	17,146
43	20,59	Caffeoylquinic Acid	13713009	232,679	77,560
46	22,34	Caffeoylquinic Acid	1345468	22,830	7,610
49	23,73	Caffeoylquinic Acid	3251138	55,165	18,388
56	27,42	not identified	3262413	-	-
65	31,94	not identified	9265090	-	-
Total			43310357	716,3089	238,7696

4.2 Antioxidant capacity

The Bd extract showed higher activity in all antioxidant assays and displayed a strong radical's scavenger activity. The activities of the extract were significantly different from quercetin in all assays (DPPH: $t_{(16)} = 9.870$, $P < 0.0001$; SO: $t_{(15)} = 2.637$, $P = 0.0187$; ICA: $t_{(12)} = 6.132$, $P < 0.0001$; and NO: $t_{(18)} = 5.597$, $P < 0.0001$).

The Bd extract demonstrated significantly higher activity in scavenging the SO radical when compared to quercetin. Table 2 shows the results obtained with the four trials.

Table 2. Results obtained with the DPPH, SO, ICA and NO assays. Results were expressed as EC50 (µg dw/ml) values and represent the mean ± SEM of a minimum of three independent assays.

	B. dracunculifolia	Quercetin
	Mean ± SEM (ug dw/ml)	Mean ± SEM (ug dw/ml)
DPPH	54.26 ± 14.48	6.12 ± 1.98
SO	11.09 ± 6.71	18.73 ± 4.96
ICA	2026 ± 687.44	281.17 ± 56.00
NO	348.99 ± 117.22	76.12 ± 13.89

4.3 Animal welfare

4.3.1 Body weight

The body weight of all animals increased during the experimental period (Fig. 12A, ANOVAone-way: $F_{(3,19)} = 4.797$, $P = 0.0144$). However, *post-hoc* tests show Bd100 animals gained less body weight than ARTH animals.

The results show a negative peak at week 6 that corresponds to the beginning of the gavage, and a recovery of the weight gain soon after (Fig 12B, ANOVAone-way: $F_{(3,35)} = 0.005597$, $P = 0.9994$).

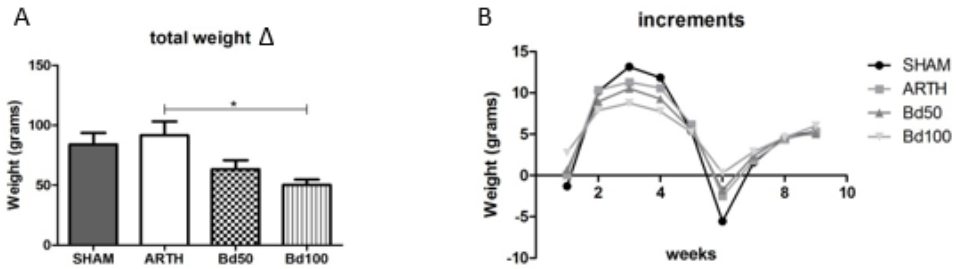

Figure 12. Evaluation of body weight. (A) Total weight gain (animal weight at the end of experiment - animal weight at the beginning of experiment). (B) Weekly Weight gain throught out the experimental period. The negative peak on week 6 corresponds to the beginning of the gavage. SHAM: animals injected with saline in the right knee and administered PBS; ARTH: animals injected with kaolin/carrageenan in the right knee and administered PBS; Bd50: animals injected with kaolin/carrageenan in the right knee and administered 50mg/kg of extract; Bd100: animals injected with kaolin/carrageenan in the right knee and administered 100mg/kg of extract. Results are expressed as mean ± SEM. *$P < 0.05$.

4.3.2 Histopathological analysis of internal organs

Regarding the weight of the internal organs, the statistical analysis shows no significant differences between experimental groups in what concerns the kidneys (Fig. 13A; ANOVAone-way: $F_{(3,19)} = 1.004$, $P = 0.4166$), the spleen (Fig. 13B; ANOVAone-way: $F_{(3,19)} = 0.2077$, $P = 0.8896$), the liver (Fig. 13C; ANOVAone-way: $F_{(3,19)} = 1.069$, $P = 0.3901$), the heart (Fig. 13D; ANOVAone-way: $F_{(3,19)} = 1.280$, $P = 0.3150$), the thymus (Fig. 13E; ANOVAone-way: $F_{(3,19)} = 0.9750$, $P = 0.4290$), the adrenals (Fig. 13F; ANOVAone-way: $F_{(3,19)} = 0.3499$, $P = 0.7897$) and the lungs (Fig. 13G; ANOVAone-way: $F_{(3,19)} = 1.186$, $P = 0.3464$).

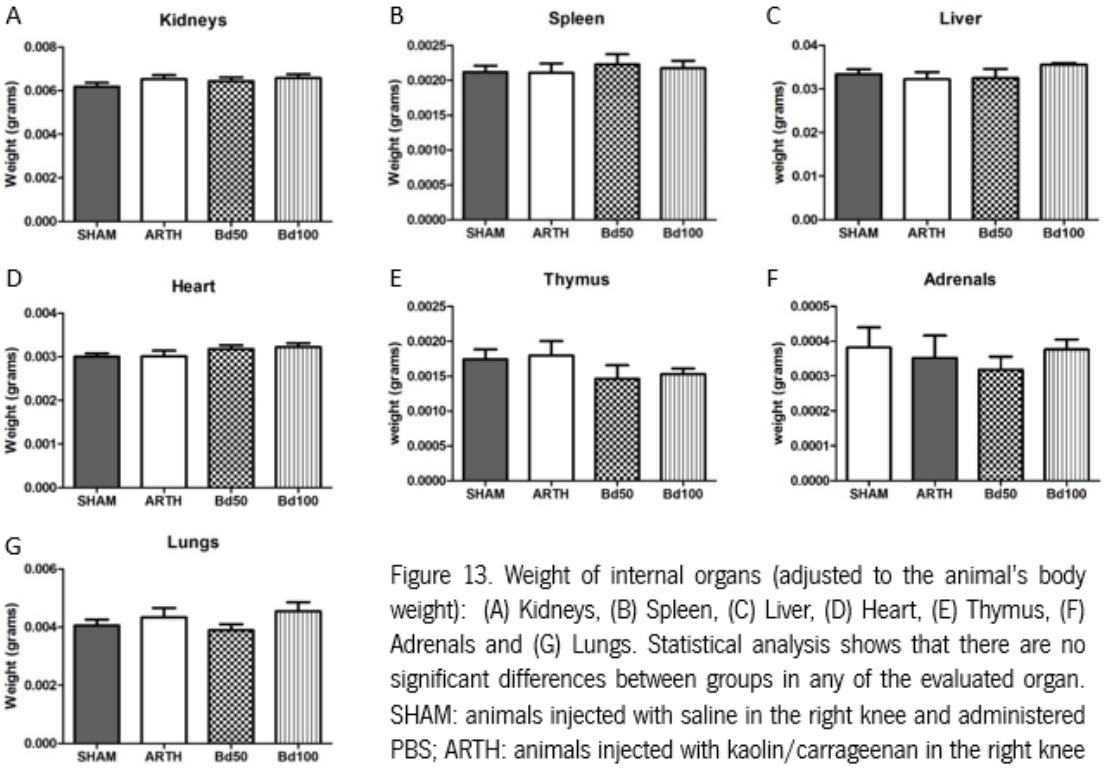

Figure 13. Weight of internal organs (adjusted to the animal's body weight): (A) Kidneys, (B) Spleen, (C) Liver, (D) Heart, (E) Thymus, (F) Adrenals and (G) Lungs. Statistical analysis shows that there are no significant differences between groups in any of the evaluated organ. SHAM: animals injected with saline in the right knee and administered PBS; ARTH: animals injected with kaolin/carrageenan in the right knee and administered PBS; Bd50: animals injected with kaolin/carrageenan in the right knee and administered 50mg/kg of extract; Bd100: animals injected with kaolin/carrageenan in the right knee and administered 100mg/kg of extract. Results are expressed as mean ± SEM.

4.4 Animal behavior

4.4.1 Pressure aplicattion measurement (PAM)

In the PAM test, the ANOVA test showed a main effect of groups for the limb withdrawal latency (LWT) (Fig. 14, ANOVAone-way: $F_{(3,19)} = 4.066$, $P = 0.025$).

ARTH animals exhibited a two-fold decrease in withdrawal threshold when compared to SHAM animals (T-test: $t_{(8)} = 4.066$, $P = 0.0031$). Post hoc comparisons revealed LWT was increased after

treatment with Bd50 significantly reversing LWT (P<0.05) and Bd100 partially reversing LWT when compared to ARTH animals.

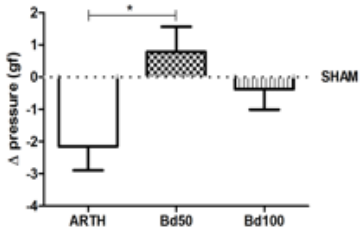

Figure 14. Evaluation of LWT through the pressure application measurement test. The LWT of the arthritic (ARTH) group was significantly decreased when compared to results in control (SHAM) animals. Bd50 reverts and Bd100 partially reverts mechanical hyperalgesia. SHAM: animals injected with saline in the right knee and administered PBS; ARTH: animals injected with kaolin/carrageenan in the right knee and administered PBS; Bd50: animals injected with kaolin/carrageenan in the right knee and administered 50mg/kg of extract; Bd100: animals injected with kaolin/carrageenan in the right knee and administered 100mg/kg of extract. Results are expressed as mean ± SEM. *$P < 0.05$.

4.4.2 Open field test (OFT)

The OFT behavior results are summarized in figure 15. There were significant difference in the OFT between groups (time in the center: ANOVAone-way: $F_{(3,19)} = 5.052$, $P = 0.0119$, immobility time: ANOVAone-way: $F_{(3,19)} = 3.265$, $P = 0.0489$; number of rearings: ANOVAone-way: $F_{(3,19)} = 3.379$, $P = 0.0444$; crossed squares: ANOVAone-way: $F_{(3,19)} = 3.338$, $P = 0.0459$).

In the OF arena, ARTH animals spend more time in the center of the arena ($P < 0.05$ Fig. 15A) and immobile ($P < 0.05$, Fig. 15B). However, ARTH animals exhibited less exploratory capacity (number of rearings: $P < 0.05$ Fig. 15C, number of squares crossed: $P < 0.05$, Fig. 15D and number of entrances in central area: $P < 0.05$, Fig. 15E) when compared to SHAM animals, indicating they developed an anxious-like phenotype. Bd treatment partial improve anxious-like behaviour.

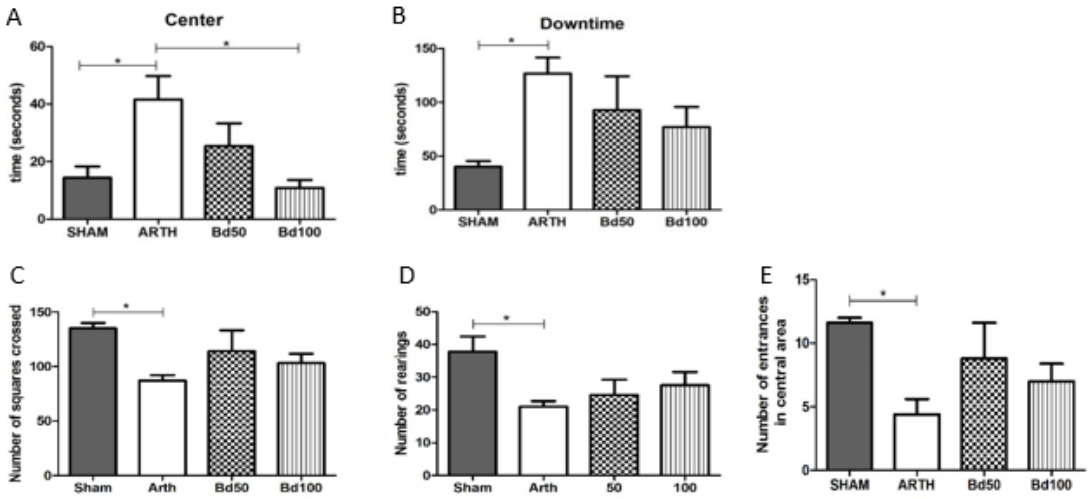

Figure 15. Behavioral assessment of anxious-like behaviour in the open field test (OFT). ARTH animals spent more time in the center (A) and more time inactive (B) and presented less exploratory capacity (the number of squares crossed (C), the number of rearings (D), and the number of entrances in the central area (E)), when compared to SHAM animals. Both Bd treatments partially revert the anxious-like behavioral. SHAM: animals injected with saline in the right knee and administered PBS; ARTH: animals injected with kaolin/carrageenan in the right knee and administered PBS; Bd50: animals injected with kaolin/carrageenan in the right knee and administered 50mg/kg of extract; Bd100: animals injected with kaolin/carrageenan in the right knee and administered 100mg/kg of extract. Results are expressed as mean ± SEM. *$P < 0.05$.

4.4.3 Forced swimming test (FST)

The learned helplessness is a measure of depressive-like behaviour and was evaluated using the FST. The ANOVA analysis showed significant differences between group concerning latency to immobility (Fig. 16A, latency: ANOVAone-way: $F_{(3,19)} = 5.268$, $P = 0.0102$), time spent struggling and swimming (Fig. 16B, struggling: ANOVAone-way: $F_{(3,19)} = 4.614$, $P = 0.0165$ and Fig. 16C, swimming: ANOVAone-way: $F_{(3,19)} = 7.408$, $P = 0.0025$) and immobility time (Fig. 16D, immobility time: ANOVAone-way: $F_{(3,19)} = 3.817$, $P = 0.0308$).

The post hoc tests revealed OA decreased latency to immobility ($P < 0.05$), the time spent swimming ($P < 0.005$) and struggling ($P<0.05$) and increased immobility time ($P < 0.05$). The administration of Bd treatments lead to an increase in the latency ($P < 0.05$ in treatment of 50 mg/Kg) and activity time (struglling and swimming: $P < 0.05$ in treatment of 100 mg/Kg) and, consequently, a decrease in the time spent immobile.

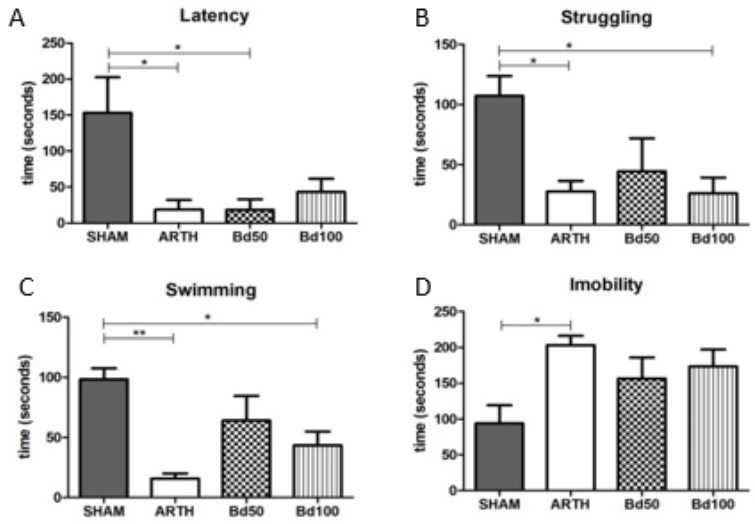

Figure 16. Behavioural assessment of depressive-like behaviour in the forced swimming test (FST). Latency to immobility (A) is significantly lower in ARTH animals than in SHAM animals. ARTH animals spent less time struggling (B) and swimming (C) than SHAM animals and spent more time immobile (D). Bd50 treatment partially reverted behavioral impairment in struggling, swimming and in time spent immobile and Bd100 treatment partially reverted behaviour in latency to immobility and in immobility time. SHAM: animals injected with saline in the right knee and administered PBS; ARTH: animals injected with kaolin/carrageenan in the right knee and administered PBS; Bd50: animals injected with kaolin/carrageenan in the right knee and administered 50mg/kg of extract; Bd100: animals injected with kaolin/carrageenan in the right knee and administered 100mg/kg of extract. Results are expressed as mean ± SEM. $*P < 0.05$, $**P < 0.005$.

4.4.4 Sucrose preference test (SPT)

The analysis of the SPT showed significant differences in the baseline values between experimental groups (Fig. 17, ANOVAone-way: $F_{(3,19)} = 13.62$, $P = 0.0001$). The ARTH group displayed decreased preference for the sweet solution in relation to the SHAM group. Conversely, sucrose preference increased with Bd treatments, thus reversing anhedonia especially in the 100 mg/kg treatment ($P < 0.001$).

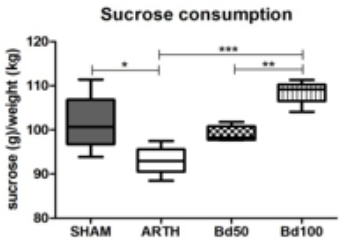

Figure 17. ARTH animals displayed an anhedonic-like behaviour as shown by the significant decrease in the preference for sucrose solution in the SPT. Bd treatments reverted the anhedonic phenotype. Values of sucrose consuption were adjusted to the animal weight. SHAM: animals injected with saline in the right knee and administered PBS; ARTH: animals injected with kaolin/carrageenan in the right knee and administered PBS; Bd50: animals injected with kaolin/carrageenan in the right knee and administered 50mg/kg of extract; Bd100: animals injected with kaolin/carrageenan in the right knee and administered 100mg/kg of extract. Results are expressed as mean ± SEM. $*P < 0.05$, $**P < 0.005$, $***P < 0.001$.

4.5 Microglia activation

In attempt to evaluate microglia activation in different areas involved in pain processing, a quantification of moderately and highly activated microglia cells (Fig. 18) in the PFC, AMY and PAG was performed.

In the PFC, the ANOVA analysis showed significant differences between experimental groups concerning moderately (PFC-CC: $ANOVA_{one\text{-}way}$: $F_{(3,15)} = 10,12$, $P = 0,0013$; PFC-PLC: $ANOVA_{one\text{-}way}$: $F_{(3,15)} = 7,450$, $P = 0,0045$; PFC-ILC: $ANOVA_{one\text{-}way}$: $F_{(3,15)} = 7,634$, $P = 0,0041$) and total activated microglia (PFC-CC: $ANOVA_{one\text{-}way}$: $F_{(3,15)} = 30,98$, $P < 0.0001$; PLC: $ANOVA_{one\text{-}way}$: $F_{(3,15)} = 11,02$, $P = 0.0009$; PFC-ILC: $ANOVA_{one\text{-}way}$: $F_{(3,15)} = 8.575$, $P = 0.0026$) (Fig. 19). In highly activated microglia there were no significant differences between groups (Fig. 19, PFC-CC: $ANOVA_{one\text{-}way}$: $F_{(3,15)} = 2.178$, $P = 0.1435$; PFC-ILC: $ANOVA_{one\text{-}way}$: $F_{(3,15)} = 0,6799$, $P = 0.5810$; PFC-PLC: $ANOVA_{one\text{-}way}$: $F_{(3,15)} = 0,7896$, $P = 0.5226$).

In the PFC, *post-hoc* tests showed ARTH animals displayed an increase in the number of moderately activated cels in the CC ($P < 0.005$) and in ILC ($P < 0.05$) and an increase in the total number of activated cells in all PFC areas (CC: $P < 0.001$; PLC: $P < 0.05$; ILC: $P < 0.05$), when compared to SHAM animals. The number of moderately activated microglia was decreased after Bd treatments. In the PFC-CC, Bd100 treatment reversed microglia activation ($P < 0.005$) while in the PFC-PLC and PFC-ILC both treatments decreaded microglia activation ($P < 0.05$ and $P < 0.005$, respectively). Both administered treatments also reversed the total microgliosis in all areas (Bd50 and Bd100, CC: $P < 0.001$; PLC: $P < 0.005$; ILC: $P < 0.005$).

In relation to the AMY (Fig. 20), ANOVA analysis showed significant differences between groups in moderately (ANOVAone-way: $F_{(3,15)} = 20.34$, $P < 0.0001$) and in total microglia cells (ANOVAone-way: $F_{(3,15)} = 19.06$, $P < 0.0001$) but not in higly activated microglia (ANOVAone-way: $F_{(3,15)} = 2.122$, $P = 0.1508$). *Post-hoc* tests demonstrate ARTH animals display more moderately and total activated microglia cells ($P < 0.001$ in both cases) than SHAM animals, an effect reversed by both treatments inverted this increased ($P < 0.001$ in both treatments).

ANOVA analysis showed there were significant differences in microglial activation in both the DLPAG (moderatly activated: ANOVAone-way: $F_{(3,15)} = 36,65$, $P < 0,0001$; highly activated: ANOVAone-way: $F_{(3,15)} = 7,774$, $P = 0.0038$; total microglia cells: ANOVAone-way: $F_{(3,15)} = 36,98$, $P < 0.0001$) and the LPAG (moderatly activated: ANOVAone-way: $F_{(3,15)} = 44,94$, $P < 0.0001$; highly activated: ANOVAone-way: $F_{(3,15)} = 7,185$, $P = 0,0051$; total microglia cells: ANOVAone-way: $F_{(3,15)} = 48,56$, $P < 0,0001$) (Fig. 21).

The *post-hoc* shoewed ARTH animals exhibit an increased microglia activation in both areas (DLPAG and LPAG) and in both cells types (moderately activated: $P < 0.001$; and highly activated cells: $P < 0.005$) and, consequently, in the total number of activated cells ($P < 0.001$). Bd treatment reversed the number of microglia cells moderately activated () and the total number of microglia activated ($P < 0.001$ in both areas) and Bd50 reversed the number of highly activated cells ($P < 0.05$ in both areas).

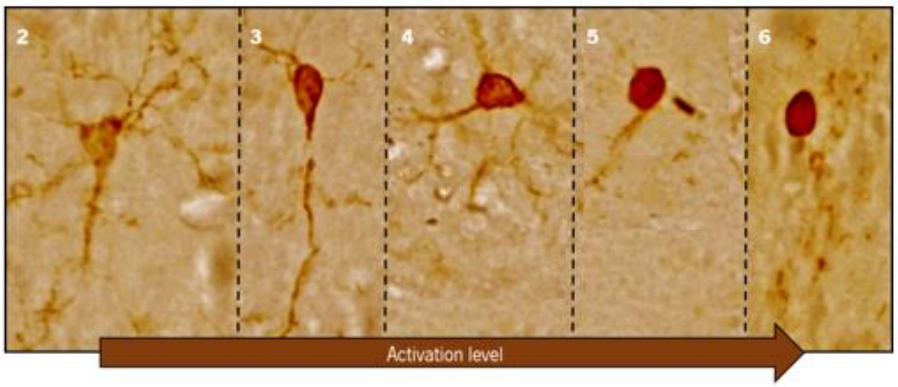

Figure 18. Photomicrographs of the different stages of the activated microglia (40x magnification). Microscope observation shows rats displayed microglia cells in all different stages of activation, but the occurrence of moderately (stages 2-4) activated cells was more common.

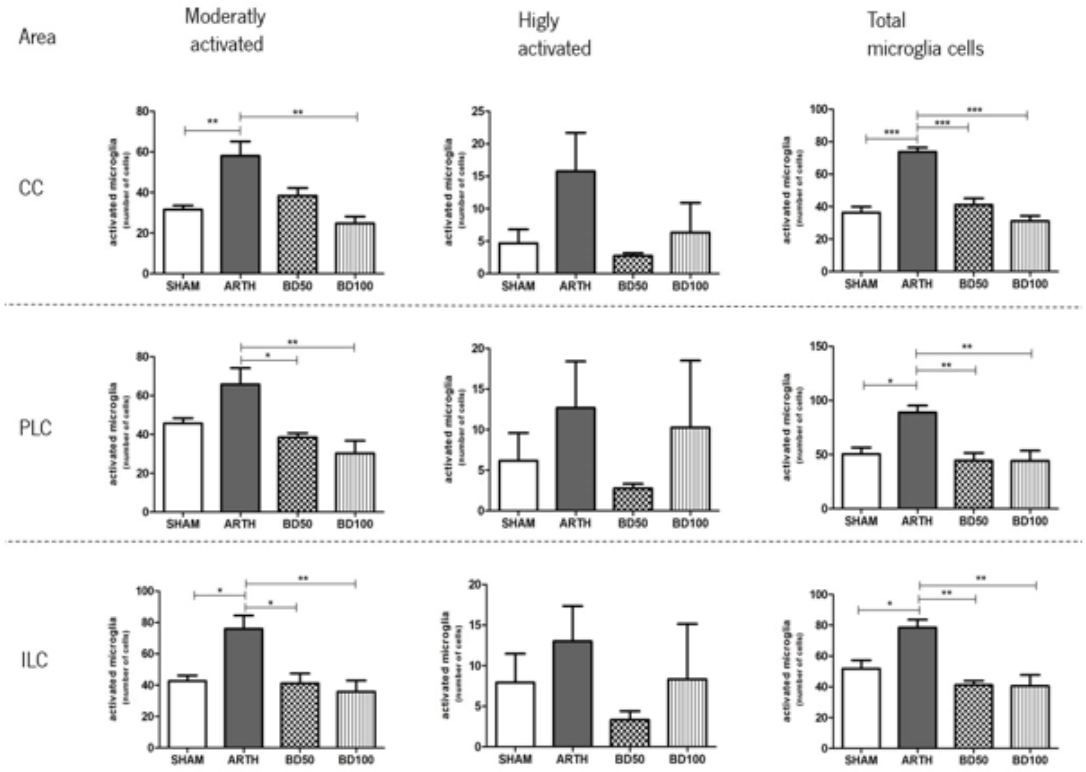

Figure 19. Number of activated microglia cells in the PFC (Prefrontal Cortex) assessed by immunohistochemistry staining with IBA-I. ARTH animals displayed an increase in the number of microglia activated cells in all areas evaluated: CC (Cingulate Cortex),PLC (Prelimbic Cortex) and ILC (Infralimbic Cortex). Both treatments reversed microgliosis. SHAM: animals injected with saline in the right knee and administered PBS; ARTH: animals injected with kaolin/carrageenan in the right knee and administered PBS; BD50: animals injected with kaolin/carrageenan in the right knee and administered 50mg/kg of extract; BD100: animals injected with kaolin/carrageenan in the right knee and administered 100mg/kg of extract. Results are expressed as mean ± SEM.* $P < 0.05$, ** $P < 0.005$, *** $P \leq 0.001$.

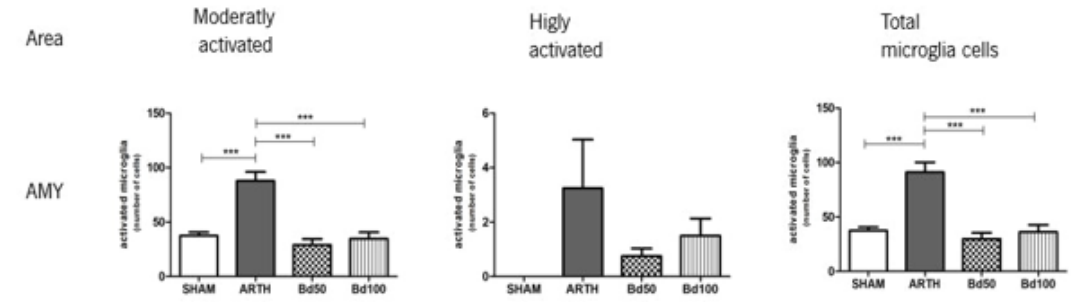

Figure 20. Number of activated microglia cells in the AMY (amygdala) assessed using immunohistochemistry staining with IBA-I. ARTH animals displayed an increase in the number of microglia activated cells and both Bd treatments reversed microgliosis. SHAM: animals injected with saline in the right knee and administered PBS; ARTH: animals injected with kaolin/carrageenan in the right knee and administered PBS; Bd50: animals injected with kaolin/carrageenan in the right knee and administered 50mg/kg of extract; Bd100: animals injected with kaolin/carrageenan in the right knee and administered 100mg/kg of extract. Results are expressed as mean ± SEM.*** $P \leq 0.001$.

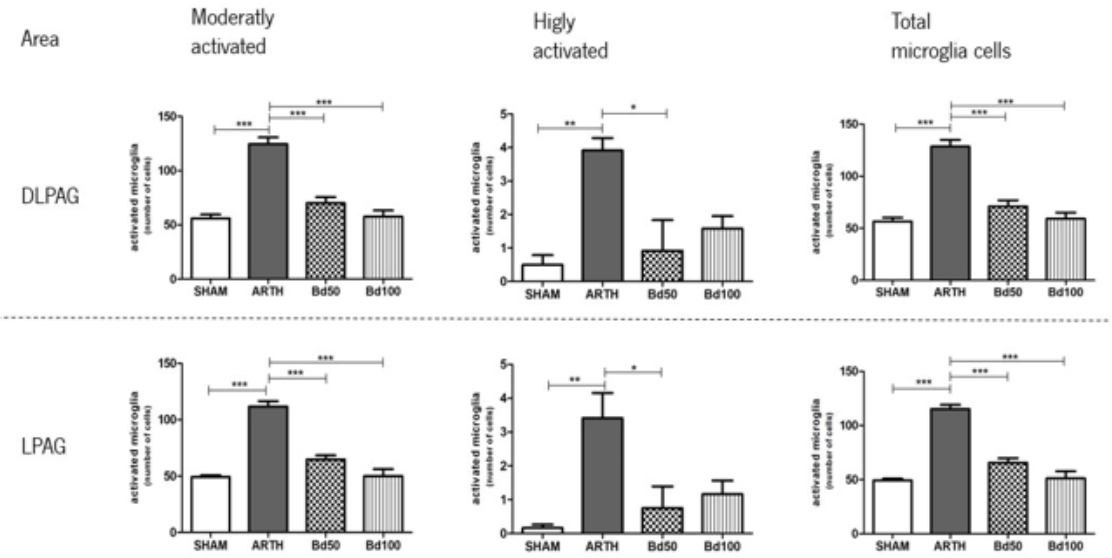

Figure 21. Number of activated microglia cells in the PAG assessed using immunohistochemistry staining with IBA-I. ARTH animals displayed an increase in the number of microglia activated cells in all areas evaluated: DLPAG (Dorsolateral Periaquedutal Grey Matter) and LPAG (Lateral Periaquedutal Grey Matter). Both Bd treatments reversed microgliosis. SHAM: animals injected with saline in the right knee and administered PBS; ARTH: animals injected with kaolin/ carrageenan in the right knee and administered PBS; Bd50: animals injected with kaolin/ carrageenan in the right knee and administered 50mg/kg of extract; Bd100: animals injected with kaolin/ carrageenan in the right knee and administered 100mg/kg of extract.Results are expressed as mean ± SEM.* $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$.

5.1 Technical considerations

5.1.1 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is a popular technique for analytical and preparative separations of compounds of interest in chemical, biological, pharmaceutical, food and biomedical sciences (Dias *et al.*, 1999). It is used in analytical chemistry to separate, identify and quantify the constituents of the mixture (Siddiqui & Noorulla, 2016).

The sample is dissolved in a solvent and introduced under a high pressure into the chromatographic column with the stationary phase. This makes the components analysis much faster and allows better separation of the compounds on the mixture with a great degree of reproducibility (Elliott & Hale, 1998). Though the initial cost for such automated apparatus may be high, the running costs are often low. A further disadvantage of the HPLC is its sensitivity as interference by sample or solvent contaminants is possible and may result in artefacts or in an apparently successful method being unusable in routine analysis (Bird, 1989).

The solvent is pumped at a constant rate and moves the components of the mixture through the column. These are distributed between the two phases according to their affinities. The nature of stationary phase is polar and the mobile phase is non-polar. In this technique, non-polar compounds travel faster and are eluted first because of the lower affinity between the non-polar compounds and stationary phase. Polar compounds are retained for longer time thus taking more time to elute (Siddiqui & Noorulla, 2016).

The time it takes for a compound to be eluted from the column is called retention time and is considered a distinguishing property of a compound under a certain elution program. When leaving the column, the components pass through a Diode Array Detector (DAD) giving rise to an electrical signal, that are recorded as a sequence of peaks thus constituting a chromatogram of the sample (Liu *et al.*, 2007; Tuzimski & Sobczynski, 2009).

5.1.2 Antioxidant activity

Most antioxidant methodologies employ the same principle where a synthetic radical is generated and the ability of a sample to eliminate or neutralize the radical is monitored by a UV/visible spectrophotometer. These methods are relatively simple based invariably on the bleaching ability of the sample.

In several cases, these studies aim at the rapid screening of large numbers of molecules, or the testing of natural extracts of complex or unknown composition endowed with some potential antioxidant activity. It is an usual and extremely necessary tools in the initial selection of substances that can be used as drugs, helping researchers to evaluate their bioactivity (Amoratti & Valgimigli, 2015)

Nevertheless, the lack of standardization in the sample preparation, reaction conditions, analytical protocols, and expression of antioxidant action make it difficult to compare results between laboratories (Xie & Schaich, 2014).

The majority of herbal preparations are designed for oral use and, therefore, the effect of exposure to the conditions of the gastro-intestinal tract must be taken into account when examining the antioxidant activity of plant extracts as lower pH conditions may alter the stability of the bioactive compounds (Keating *et al.*, 2014). A compound that exhibits low antioxidant activity *in vitro* is likely to exhibit very little activity *in vivo* (Halliwell, 1995), however the methods used to assess antioxidant capacity do not support the existence of a biological effect *in vivo* (Amoratti & Valgimigli, 2015).

5.1.3 The rat as an animal model

The laboratory rat has been used as an animal model for physiology, pharmacology, toxicology, nutrition, behavior, immunology and neoplasia for over 150 years (Aitman *et al.*, 2008). Because of its size, ease of manipulation and breeding, it remains one of the preferred choices for most research fields throughout the twentieth century (Aitman *et al.*, 2008). There are many reasons why the rat is the one of most commonly selected subjects for research in mammalian neuroscience. Firstly, rats are not too small for accurate stereotaxic localization of discrete brain areas and they are cost-effective (Paxinos & Watson, 1997). Second, rats are generally hardy animals, resistant to infections (Paxinos & Watson, 1997). Third, a number of inbred strains are available commercially (Paxinos & Watson, 1997).

The rat model allows the study of biochemical changes that influence nociceptive behavior and provide new opportunities to translate basic research to human patients. However, there are several discrepancies when comparing results of animal models with those of humans. The homologies between rats and primates are based on the pattern and density of neuronal connections, anatomical and functional properties of neurons and cerebral cytoarchitecture (Sereno & Tootell, 2005; Wallis, 2012). However, identifying functions of frontal cortex in one

species could allow the extrapolation of common mechanisms to the frontal cortices of other species. In this sense, studies of rat prefrontal cortical function do not model primate prefrontal cortical function per se, but form a separate line of investigation that will converge with research in primates (Brown & Bowman, 2002).

Another question is that many different designations are still used to describe a single structure, and in some cases, the same term is used for completely different structures. In addition, it is important to highlight there are also differences in the delimitations of the brain areas between species (Paxinos & Watson, 1997).

5.1.4 The experimental model of OA

OA in humans and other species may not be a single disease but rather a syndrome with a characteristic pattern of clinical signs (pain, disability) and pathological changes in joint tissues (Little & Zaki, 2012). Typically, OA comprises cartilage erosion, thickening of subchondral bone, decreased mineral density, excessive marginal new bone formation (osteophytes), synovitis and joint capsule thickening/fibrosis (Little & Zaki, 2012). Consequently, an animal model of OA should try to mimic the human pathology (Little & Zaki, 2012).

Unfortunately, there is no single ideal experimental model of OA. Thus, in selecting the most appropriate model, several factors should be taken into account regarding the aim of the study, as well as its budget and the technical equipment available (Lampropoulou-Adamidou *et al.*, 2013). Pathogenetic studies may require the use of naturally occurring models of OA, while molecular studies often use genetic models. For therapeutic studies, the use of surgical models is the most appropriate (Lampropoulou-Adamidou *et al.*, 2013).

The intra-articular injection of monoiodoacetate (MIA) is the most used in OA pain research (Little & Zaki, 2012). However, the intra-articular injection of monoiodoacetate induces acute cartilage degradation and joint pain with its effects being however too extensive when compared to early stage human OA (Barve *et al.*, 2007; Amorim *et al.*, 2015). Also, considerably studied are surgical/traumatic models, such as the medial meniscus transection, considered relevant in the context of the impact upon articular structures and pain but limited in terms of the inflammatory component of OA (Bendele, 2001).

In this work, we injected a mixture of kaolin and carrageenan in the right knee. OA induced through the injection of kaolin and carrageenan into the synovial cavity of one knee joint results in damage

to the cartilage, inflammation of the synovia and synovial fluid exudates, as well as pain and neuroplastic changes in the peripheral and central nervous system (Neugebauer, 2013).

Although the K/C model has been defined as an acute monoarthritis model, recent evidence (Amorim et al., 2014) shows this animal model can be used as a model of OA as 4 weeks after induction, histologic analysis shows a degeneration of the femoral plateaux of the joint, fibrillation and subchondral sclerosis.

As shown by Amorim *et al.* (2014), K/C-induced OA is a slowly developing and use-dependent degeneration of the knee joint translated as edema, inflammatory reaction of articular structures, cartilage thinning, focal disorganization of chondrocytes, sclerotic bone and development of cysts, changes concomitant with the development of human OA grade 4. In addition, animals also develop mechanical hyperalgesia and emotional impairments, both important features of the human condition. The advantages of this model also include a slower onset and progression and its restriction to a single joint (Amorim *et al.*, 2015).

Nonetheless, further studies involving a time dependent evaluation of the succession of events affecting knee joint structure are needed for this K/C-induced arthritis to be considered a valid osteoarthritis model (Amorim *et al.*, 2014).

5.1.5 Anesthesia

According to American Society of Anesthiologists (2006) anesthesia is defined as a continuous process of progressive central nervous system (CNS) depression and its effects are usually described as the combination of three main factors: hypnosis, analgesia and muscle relaxation.

Usually a combination of different drugs is used to achieve all these effects with an adequate margin of safety (Arras *et al.*, 2001) thus reducing the risk of negative side effects, such as hemodynamic instability (Arras *et al.*, 2001; Tonner, 2005).

A combination of ketamine/medetomidine is often used in many species, including laboratory animals (Cruz *et al.*, 1998). The administration of a combination of the two drugs not only substantially decreases the dose required to induce anesthesia when compared to the effect of each drug alone but decrease their side effects (Cullen, 1996; Sun *et al.*, 2003). In addition, this combination constitutes an efficient anesthetic plan in small laboratory animals (mice and rats), with hemodynamic stability and muscle relaxation. An additional advantage is its intraperitoneal injection administration, which is a simple procedure that causes little discomfort to the animal

(Cruz *et al.*, 1998; Jang *et al.*, 2009). Rare negative side effects include cardiovascular and respiratory depression, hypothermia, hyperglycemia and diuresis (Sophocleous & Idris, 2014).

Finally, anesthesia using ketamine/medetomidine can be reversed using a specific antagonist, atipamezole, shortening the recovery time and decreasing potential side effects (Hu *et al.*, 1992; Cruz *et al.*, 1998).

5.1.6 Ovariectomy

Sex hormones have an important role in the regulation of bone mass and turnover in adult and ageing skeleton (Sophocleous & Idris, 2014). Oestrogen belongs to the gonadocorticoid class of steroid hormones and is responsible for the development of female secondary sexual characteristics, the regulation of menstrual cycle, the timing of ovulation in pre-menopausal women and maintenance of pregnancy (Sophocleous & Idris, 2014).

The skeleton is one of the main targets of oestrogen, as it regulates bone growth and remodeling in both men and women (Sophocleous & Idris, 2014). Oestrogen is critical for normal brain function and its depletion after menopause accounts, at least in part, for cognitive decline, anxiety and hypertension (Patki *et al.*, 2013). In fact, estrogen is considered protective against anxiety, known to restore learning-memory function and reported to help maintain normal blood pressure in females (Williams *et al.*, 2010; Patki *et al.*, 2013). By contrast, estrogen reduction during menopause was reported to be linked to increased oxidative stress (Patki *et al.*, 2013). Preventive effects of drugs mimicking antioxidant effects were reported to attenuate high blood pressure and prevent heightened anxiety-like behavior. Oxidative stress was considered causal to these behaviors (Patki *et al.*, 2013).

Oestrogen deficiency, however, could be reached at an earlier stage either by chemically inducing ovarian failure, using the chemical 4-vinylcyclohexene diepoxide (VCD), by suppression of the hypothalamus-pituitary-ovarian axis through the administration of the gonadotropin-releasing hormone agonist, buserelin, or by surgically removing the ovaries, a procedure referred to as ovariectomy (Sophocleous & Idris, 2014).

VCD treatment in rodents leads to a gradual onset of ovarian failure, with hormonal and cyclic changes, which mimic the pre-menopausal transition in women. Ovariectomy, on the other hand, has been criticized has it interrupts ovarian function rather markedly, leading to an abrupt hormonal loss instead of a gradual decline, which naturally happens in women (Sophocleous & Idris, 2014).

Several ovariectomy protocols are available and the choice of which surgical approach to use is very important (Lasota & Klonowska, 2004). The abdomen of rodent can be accessed through three different methods: (i) a single surgical incision made on the dorsal midline at the caudal edge of the ribcage; (ii) a double (bilateral) dorsolateral incisions and (iii) a single ventral transverse incision on the middle part of the abdomen (Sophocleous & Idris, 2014).

In the ventral approach, the suture will be in direct and constant contact with the bedding of the cage, increasing the chances of post-operative infections. In addition, the incision is subjected to the weight of the viscera, with evisceration and postsurgical hernia occurrence being potential sode effects (Sophocleous & Idris, 2014). For these reasons, a dorsal approach for performing ovariectomy is recommended. The single midline skin incision technique is preferred because it is technically easier and less time consuming (Lasota & Klonowska, 2004).

5.1.7 Gavage intragastric administration

The simplest method for administration of a drug is to include it in the food or drinking water (Nebendahl, 2000). However, this is not practicable with drugs that are unpalatable, insoluble or chemically unstable in drinking water, when they irritate the mucosa of the gastrointestinal tract (Nebendahl, 2000) or when there's need to warranty right the amount of drug is administered to an animal (Shimizu, 2004; Vandenberg *et al.*, 2014). A ball tip needle was used to prevent damage to the esophagus and to ease the passage through the glottal opening to the trachea (Shimizu, 2004).

However, gavage does not completely model the human dietary exposures. Drug administration via the diet, allows it to interact with numerous surfaces in the oral cavity including the buccal, sublingual, gingival, palatal and labial mucosa (Madhav *et al.*, 2012), where absorption is possible an effect that does not occur when using gavage.

Moreover, experiments designed to assess the effects of gavage indicate that the process of gavaging animals induces rapid, pronounced and statistically significant effects on stress-related responses (Vandenberg *et al.*, 2014). Individual studies have shown gavage can increase the secretion of the stress-response hormone corticosterone in mouse feces (corticosterone is secreted by the adrenal gland as a result of activating the hypothalamic-pituitary-adrenal axis by stress) (Walker *et al.*, 2012). Other studies indicate gavage leads to increased heart rate, blood pressure and glucocorticoid concentration that persist for 30 to 60 minutes following the event. These

responses suggest that, despite their routine use in laboratory studies, this procedure is acutely stressfull for animals (Balcombe *et al.*, 2004). Interestingly, the effects on corticosterone levels vary depending on the vehicle and volume used (Bonnichsen *et al.*, 2005*;* Vandenberg *et al.*, 2014). In addition, solvents may also influence the effect of the extract administered (Shimizu, 2004). In this work, the solvents and vehicles used (PBS and Tween 20) in the gavage were found to be suitable in most instances and were shown not to affect drug action (Shimizu, 2004).

5.1.8 Animal behavior analysis

5.1.8.1 Pressure application measurement (PAM)

The PAM test is a preclinical test that allows the evaluation of hypersensitivity in animals and is often used in osteoarthritis trials (Barton *et al.*, 2007). However, some authors report some inaccuracy regarding the values obtained due to variations caused by the placement of the operator's finger on the sensor (Barton *et al.*, 2007). Thus, there is always variation associated with the placement of the sensor on the knee joint of the animal to be tested which can also vary from operator to operator (Barton *et al.*, 2007; Bagüés *et al.*, 2016).

Besides being highly dependent on the expertise of the experimenter it requires the handling of animals while performing the test. In our work, our results were consistent and the PAM test was a useful tool for the study of experimental OA.

However, there are circumstances during which there can be little doubt an animal is feeling pain—notably when it is responding to stimuli through vocal responses such as squealing or groaning. On the other hand, it is far more difficult to certify that at a given moment, an animal feels no pain because it is presenting no typical physical signs or overt behaviors. For this reason, while the application of acute noxious stimuli results in the display of a series of consistent behavioural responses, its absence does not demonstrate the animals are not suffer of pain (Mogil, 2009).

Consequently, nociceptive behaviour is mostly assessed by observing physical reactions of animals to a stimulus known to cause pain in humans although the assessment of other impairments, such as constant pain and imbalanced of sleep patterns and mood, might better reflect the diverse symptomatology of chronic pain in humans (Blackburn- Munro, 2004).

5.1.8.2 Open field test (OFT)

The open field test was originally described by Hall (1934) for the study of emotivity in rats. The procedure consists of subjecting an animal, usually a rodent, to an unknown environment from which escape is prevented by surrounding walls. Open field testing is currently one of the most popular procedures in animal psychology (Hall, 1934; Prut & Belzung, 2003; Thompson *et al.*, 2015).

The open field has become so popular that its use has been extended to a great number of species. As a result, several different versions are used, differing in the shape of the environment (circular, square or rectangular), the lighting (lighting from above with a bulb above the open field or lighting from underneath with a bulb placed under a transparent floor, sometimes red light is used), the presence of objects within the arena such as platforms, columns, tunnels and others (Prutt & Belzung, 2002).

In this test, rodents tend to avoid open and illuminated spaces that can expose them to predation, spontaneously preferring the periphery of the apparatus rather than the central parts. Decreased time spent in the central area as well as low exploratory capacity may indicate anxiety behaviors (Thompson *et al.*, 2015). Anxiety-like behaviour in the open field is triggered by two factors: individual testing (the animal is separated from its social group) and agoraphobia (the arena is very large in relation to the animal and their natural environment) (Prut & Belzung, 2003).

Tests for assessing anxiety responses in rats include the open field test, the elevated plus-maze test, the elevated zero-maze test and the light/dark box. However, each test for anxiety-like behaviour comes with idiosyncrasies and limitations and no one test provides the ideal model of anxiety (Cryan & Holmes, 2005).

The differences in animals' locomotor activity could distort other behavioural measures (Stanford, 2007) and visual acuity can also confound performance on the OFT (Cryan & Holmes, 2005). It would also be desirable to score during the night, while animals are in the active phase of their diurnal cycle and so actually more active (Prutt & Belzung, 2002; Stanford, 2007).

5.1.8.3 Forced swimming test (FST)

The FST was designed by Porsolt in the 1970s as a primary screening test for antidepressants. At present, it is still one of the best models for this procedure (Cryan &Holmes, 2005) and numerous studies have demonstrated clinically relevant pharmacological validity in this test (Goffer *et al.*,

2013). In this paradigm, the animal faces an aversive scenario from which it is impossible to escape (Porsolt *et al.*, 1978). The test leads to a condition known as learned helplessness, an important component of depression (Castagné *et al.*, 2010).

The animal's perseverance to escape from the situation is inversely correlated with the development of depressive-like behavior (Porsolt *et al.*, 1978; Wang *et al.*, 2017). It is also very reliable to test the potential of new antidepressant treatments (Porsolt *et al.*, 1977; Porsolt *et al.*, 1978).

The immobility is the most important variable related with the depressive-like behaviour. This behavior is interpreted in the literature as an index of behavioral despair since antidepressant drugs are able to reduce the time spent in this posture (Saenz *et al.*, 2006; Goffer *et al.*, 2013). Then, the most important behavior was climbing and secondly diving. Both behaviors were inversely correlated with the behavioral despair and therefore with the immobility. Diving was considered as a behavioral emergent pattern within the FST, since it is not a typical behavior of FST and is infrequently reported because diving frequencies are very low and its distribution is not homogenous between the groups (Saenz *et al.*, 2006).

Although the biological meaning of climbing and diving are not well understood, they seem to represent different escape responses, where diving could be more functional than climbing, in terms of its effectiveness (Saenz *et al.*, 2006). In our study, we measured time of immobility, latency to immobility, time spent in swimming and time spent in struggling, that is a total time spent in diving and climbing.

This test presents as an advantage its low cost besides being is extensively validated (Wang *et al.*, 2017).

Nevertheless, FST have some limitations like the duration of immobility observed in a particular experiment depends on a number of factors, including the animal strain, the dimensions of the cylinder (immobility occurs more rapidly if the dimensions are such that the animal learns quickly that there is no escape), the depth of the water, the illumination of the experimental room, and, in the rat protocol, the duration of the test (Castagné *et al.*, 2010).

Neither the interval between the sessions in the rat (up to 4 weeks) nor the temperature of the water (between 22◦ and 35◦C) appears to influence significantly the duration of immobility in the rat behavioral learned despair test (Castagné *et al.*, 2010). However, temperatures that are colder could induce swimming behavior to increase heat and thereby fail to identify rats that are really

immobile and, on the other hand, if the testing temperature is too warm, there will be a tendency for all rats to float or become immobile (Overstreet, 2012).

Other limitations are related to the time at which the test is performed: a prolonged single exposure to water induces marked hypothermia (Castagné *et al.*, 2010). Even with relatively warm water (more than 30◦C), the body temperature rapidly decreases toward that of the water. This problem is avoided using other tests like the tail suspension procedure, where no hypothermia is observed but this test is only validated in mice (Castagné *et al.*, 2010; Wang *et al.*, 2017).

Furthermore, the rat is extremely sensitive to changes in its external environment. Any small movement by the experimenter, or by another rat in a neighboring cylinder, can cause the animal to start swimming again. Consequently, it is recommended that the experiment be performed in very quiet conditions, with constant illumination and a minimum of movement during the behavioral evaluation time. When animals are being tested in parallel, they must be visually separated by an opaque screen (Castagné *et al.*, 2010)

The SP test was also performed as it is another standard test in rodents to assess depressive-like behaviors. In fact, the SPT and FST are independent tests for depressive-like behaviors in rats as the former evaluates anhedonia and the latter learned helplessness. Importantly, when compared with the FST, the SPT has minimal requirements for motor coordination, and findings are less likely confounded by any potential motor deficits or spontaneous pain (Wang *et al.*, 2011; Goffer *et al.*, 2013).

We opted to assess both anhedonia and learned helplessness because either are fundamental features of depression, and are common to all of the depressive subtypes (i.e. typical or atypical major depression, dysthymia, or melancholia) (Anisman & Matheson, 2005).

5.1.8.4 Sucrose preference test (SPT)

Whether a sensation is pleasurable or not is determined by both the stimulus itself and the mental state of the animal experiencing it (Figueroa *et al.*, 2015). Meaning a particular food or solution may be perceived as agreeable or unpleasant according to the internal status of the individual (Figueroa *et al.*, 2015). Thus, hedonistic reactions during ingestion are affected not only by the characteristics of the food or solution, but also by the physiological state (nutritional status, disease and internal temperature) and psychological state of the animal (stress, depression and anxiety) (Figueroa *et al.*, 2015).

Animals exposed to chronic pain tend to develop behaviors similar to those of anhedonic humans. In particular, the decrease in the consumption of palatable solutions at low concentrations has been suggested as a reliable indicator of an anhedonic state (Willner *et al.*, 1987; Wang *et al.*, 2011; Goffer *et al.*, 2013). This is based on the assumption the post-ingestive and oral effects of sucrose usually activate pleasure pathways and thus generally display a higher degree of preference or acceptance that are perceived differently when an animal is anhedonic (Figueroa *et al.*, 2015).

Although the analysis of anhedonia (lack of interest in pleasure) in rodents should be approached with caution, a reduced preference for a sweet solution is considered to share some analogy with anhedonia and depressive behaviour in humans (Figueroa *et al.*, 2015) and therefore, the SPT is widely used in stress studies (Wang *et al.*, 2011; Amorim *et al.*, 2014) to assess hedonic-like behaviour in rodents (Overstreet, 2012). The advantages of this test are its low costs and that it's a fast and simple drug screening test (Wang *et al.*, 2017).

However there is a variety of SPT protocols available that differ in terms of prior deprivation of food and water, duration of the evaluation session and cycle (light/dark) in which animals are tested (Overstreet, 2012; Wang *et al.*, 2017). Other difficulties include preventing possible effects of side preference in drinking behavior and spillage (Wang *et al.*, 2017). To avoid these disadvantages, our tests were performed in the dark phase of the cycle, when rats are most active (Tõnissaar *et al.*, 2006; Franklin *et al.*, 2016).

5.1.9 Immunohistochemistry

From the histological point of view, identification and separation of microglia and macrophages is difficult, since most of the markers used are simultaneously expressed in both types of cells (Boche *et al.*, 2013).

The most commonly used markers for microglial identification are CD45, CD11b, CD68, Major Histocompatibility Complex - class II (MHC II) and Ionized calcium-Binding Adapter molecule-1 (IBA-1) (Boche *et al.*, 2013).

In the literature, MHC II, in addition to microglia, is expressed in other cells, such as B1 lymphocytes and macrophages (resident and peripheral) (Ransohoff & Cardona, 2010). On the other hand, some authors have described astrocytes as cells expressing MHC II (Lorger, 2012). Therefore, all these cell types may be labeled using the anti-MHC II Ab, together with the microglia,

thus increasing their immunohistochemical expression and, indirectly, affecting its the quantification.

The same situation occurs with anti-CD68 Ab. This is referred to as selective for cells of the myeloid hematopoietic line, such as macrophages and microglia (Holness & Simons, 1993). However, Gottfried and collaborators (2013) demonstrated their labeling in cells outside this cell line, in lymphocytes and fibroblasts, as well as, in endothelial cells and some tumor cell lines.

The calcium-binding molecule IBA-1, discovered in 1996, is a 17kDa protein consisting of 147 amino acids. The expression of IBA-1 appears to be specific for *in vitro* and *in vivo* microglia, constitutive and independent of its activation stage (showing higher marking when microglia is activated) (Ito *et al.*, 2001). The IBA-1 has been used to differentiate microglia from macrophages in tumors and other pathologies when used with other markers (Borda *et al.*, 2008; Deininger *et al.*, 2000).

The overactivated/activated stage paradigm has been increasingly studied in neurodegenerative diseases in an attempt to uncover mechanisms of immunopathogenesis, and advances in understanding of molecular and functional states of microglia (Loane & Kumar, 2016).

Activated microglia undergo a dramatic transformation including shortening and thickening of cells processes and enlargement of cells bodies. The most activated form of microglia is characterized by an ameboid appearance and absence of cells processes (Kreutzberg, 1996; Nakamura, 2002).

The transformation of microglia could be seen as such an adaptive process. Highly activated cells (induced by IFN-Ɣ) are characterized by a pro-inflammatory phenotype and secretion of neurotoxic substances (e.g., NO, glutamate) (Eßlinger *et al.*, 2016). Moderately activated cells (induced by IL-4, IL-13 or TGF-β) are characterized by an anti-inflammatory phenotype and secretion of neurotrophic factors (e.g., BDNF) (Eßlinger *et al.*, 2016) and are involved in regulating brain development by enforcing the programmed elimination of neural cells, and seem to enhance neuronal survival through the release of trophic and anti-inflammatory factors. In addition, in the mature brain, microglia facilitate repair through the guiding the migration of stem cells to the site of inflammation and injury, and might be involved in neurogenesis (Block *et al.*, 2007).

The stimuli that cause microglial overactivation and dysregulation can be diverse, ranging from environmental toxins to neuronal death or damage (Block *et al.*, 2007).

5.2 Phytochemical profile of Bd extract

The HPLC spectra of Bd extract displayed characteristic absorbance and retention time for the caffeoylquinic acid analogues. Plant phenolics, and, in particular, the caffeoylquinic acids (CQAs), can synergistically or additively provide protection against damage induced by free radicals during oxidative stress, acts as a scavenger of reactive oxygen and nitrogen species, and reduce the risk of chronic diseases in humans (exhibit anticarcinogenic, antimutagenic and glucose lowering effects as well as antiobesity properties) (Arakawa *et al.*, 2009; Puangpraphant *et al.*, 2011; Marković & Tošović, 2016). The antioxidant activity of CQAs is influenced by the number, and position, of esterification on the quinic moieties (Xu *et al.*, 2012). CQAs also play key roles in increasing plant protection against harmful UV light (Clé *et al.*, 2008; Lattanzio *et al.*, 2009) as well as in resistance of plants to bacteria (Niggeweg *et al.*, 2004), virus (Lizzi *et al.*, 1995) and herbivores (Leiss *et al.*, 2009). Caffeoylquinic acids are produced as monoesters (monocaffeoylquinic acids, monoCQAs, which include chlorogenicacids) and diesters (dicaffeoylquinic acids, (diCQAs)) by members of plant families, such as Asteraceae (Lattanzio *et al.*, 2009).

Besides caffeoylquinic acid derivatives, other phenolics belonging to the flavonoid class, such as the flavones apigenin, have been identified in the Bd extract. From a quantitative viewpoint, this compound is considered a minor constituent of the total phenolics content of extract.

Apigenin is a low molecular weight polyphenolic compound and one of the most common dietary flavonoids in the human diet (Paredes-Gonzalez *et al.*, 2015). Several biological effects of apigenin are related to its antioxidant effects and its role in scavenging free radicals. Furthermore, it exhibits anti-mutagenic, antinflammatory, antiviral, and purgative effects (Shukla & Gupta, 2010). Apigenin is one of the most bioactive flavones in plants, and epidemiologic observations have shown flavone rich diets are associated with decreased risk of developing certain cancers (Chinembiri *et al.*, 2014).

Previous phytochemical studies of this plant demonstrated phenolic acids (caffeic acid, *p* coumaric acid, cinnamic acid, drupanin, baccharin and artepillin C) (de Sousa *et al.*, 2008; Rezende *et al.*, 2014; Veiga *et al.*, 2017).), hydroxycinnamic acids (3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*caffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid) (Rezende *et al.*, 2014) and flavonoid compounds (aromadendrin-4'- methyl ether) (Filho *et al.*, 2004; de Sousa *et al.*, 2008; Guimarães *et al.*, 2012) were predominant in the composition of Bd extract.

5.3 Evaluation of antioxidant activity

Free radicals are highly unstable molecules and are able to oxidize biomolecules while antioxidant metabolites can eliminate free radicals (Gulcin *et al.*, 2012). However, the use of several antioxidant activity determinations with differing reaction mechanisms is necessary to have an overall understanding of the mechanisms of action of an antioxidant (El-Abbassi *et al.*, 2012). In our experiment, we used four different assays to determine the antioxidant activity of Bd extract (DPPH, ICA, NO and SO scavenging).

The extract exhibited distinct behaviours in several tests conducted to evaluate the antioxidant activity. In the DPPH and ICA assays, lower EC50 indicates higher antioxidant activity, even more elevated than with Quercetin a pure compound. In SO and NO assays, lower EC50 meant our extract displayed a higher ability to scavenge the anion superoxide and inhibit the formation of nitric oxide, respectively. Our study, the observed antiradicalar potency of the Bd extract was considerably superior to its capacity to iron-chelation. It is noteworthy to mention that Quercetin is a pure compound, the plant extract is composed by a complex array of compounds.

Oxidative stress has been implicated in exacerbated inflammation, a process of cellular aggression mainly mediated by reactive oxygen/nitrogen species (ROS/RNS) (Silva *et al.*, 2008). Our Bd extract is a good inhibitor of NO production, an important feature since NO is a mediator of inflammation, and, at certain levels may be harmful. In addition, NO when coupled to superoxides leads to the formation of peroxinitric leading to protein damage and lipid peroxidation (Guzik *et al.*, 2003; Yang *et al.*, 2015; Weidinger & Kozlov, 2015).

Our Bd extract displays great scavenger ability of SO, an important ability as in the presence of hydrogen peroxide, superoxide gives rise to hydroxyl radical (Yang *et al.*, 2015; Duarte *et al.*, 2016). Our results suggest the Bd extract could have a beneficial role in inflammatory disorders by trapping two important mediators of inflammatory processes: nitric oxide (NO) and superoxide (SO). Previous studies reported compounds present in Bd extracts, such as caffeoylquinic acid acid and flavonoids (as apigenin), are efficient scavengers of free radicals (Sroka & Cisowski, 2003; Hatia *et al.*, 2014).

Taking into account the overall results obtained, our Bd extract displayed higher antioxidant capacity, mainly because of their phenolic composition, characterized by hydroxylated aromatic rings that act as antioxidants (Rezende *et al.*, 2014). Phenolic compounds may also contribute

directly to antioxidant action, because of their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Rezende *et al.*, 2014).

However, lack of standardization in sample preparation, reaction conditions, analytical protocols, and expression of antioxidant action make it difficult to compare results between laboratories (Xie & Schaich, 2014). In addition, seasonality influences the quantitative chemical profile of *B. dracunculifolia* (Figueiredo-Rinhel *et al.*, 2013).

Our results show the Bd extract exerts its antioxidant activity due to a sum of free radical scavenging and iron chelating activities, in accordance with data from Guimarães and collaborators (2012).

5.4 Animal welfare

5.4.1 Body weight

Body weight data showed treatment with the higher dose of Bd extract (100 mg/Kg), decreased body weight gain. Ichi *et al.* (2009) reported supplementation of green propolis reduced white adipose tissue weight and serum levels of cholesterol and triglyceride. As a possible mechanism, they proposed that propolis inhibits triglyceride absorption that results in a reduction of serum triglyceride concentration. In another study by Koya-Miyata and collaborators (2009), it was shown treatment with propolis for 10 days reduced body weight gain, weight of visceral adipose tissue, liver and serum levels of triglyceride, cholesterol, and non-esterified fatty acids in mice fed a high-fat diet. More recently, Sakai *et al.* (2017) found treatment with propolis reduces body fat content and that reduction in body fat content may be associated to increased feces weight and inhibition of fat absorption. Although propolis has a more complex composition, its major botanical component derive from Bd (Cestari *et al.*, 2011), thus it is possible the Bd extract is having a similar effect. On the other hand, some *Baccharis* species are used in folk medicine as diuretics (Retta *et al.*, 2009) and we could not exclude the possibility that Bd extract reduces body weight gain by modulating lipid and glucose metabolism in the liver.

Still, it would be important to determine (i) body fat percentage, (ii) lipid content in feces and (iii) urinary excretion of rats in order to understand what effect the Bd extract might be having.

It is noeworthy to mention that in the first week of Bd administration, all groups tested displayed a negative peak in terms of body weight gain. This effect is probably related to the initiation of gavage, rather than with the Bd extract, as this procedure might promote light nausea, discomfort and even pain and is the most stressful and technically demanding method of oral administration (Kai *et al.*, 2006). Weight increment increase henceforth further support this hypothesis.

5.4.2 Well-being

The toxicity of *Baccharis dracunculifolia* has been demonstrated in laboratory animals by Rodrigues *et al.* (2009). Clinical signs of behavioral toxicity (as lethargy, decreasing locomotor activity and exploratory behavior) were observed in some animals treated with 3×2.0 g/kg of Bd. In this work, we opted to used concentrations 40 and 20 times lower (50 and 100 mg/kg, respectively) than those used in previous experiments.

The low toxicity of *Baccharis dracunculifolia*, offers a wide margin of safety for beneficial doses. Nevertheless, further in-depth toxicity studies are needed to confirm our findings. In this respect, the present work provides some indications at what doses further efficacy studies can be conducted.

Histological analyzes were performed on the liver, kidneys, thymus, spleen and adrenals.

The thymus and spleen are the main immune organs. The thymus, a primary lymphoid organ, influences and regulates the differentiation, development and function of T lymphocytes by secreting thymic hormone. It has an important role in regulating the immune balance of the organism and maintaining the autoimmune stability. The spleen, a secondary lymphoid organ, is the body's largest lymphoid organ, which contains lymphocytes and macrophages. It is closely related to both humoral and cellular immunity, and it plays an antitumor role through a variety of mechanisms (Liu *et al.*, 2015).

The suprarenal gland constitutes a principal node of the mammalian endocrine system and play important roles in the regulation of metabolism, water and salt balance, reproduction, immune responses, and various CNS functions (Oster *et al.*, 2006).

The histological examination of cells and tissues is an important procedure in chronic toxicity evaluation (Sireeratawong *et al.*, 2016). The histological examination showed no abnormality of the internal organs of our Bd animal when compared to the control group. There were no macroscopic

or microscopic changes in the internal organs, nor changes in weight induced by the Bd treatment. Our results suggest the Bd extract had no impact on the normal function of the internal organs.

A common complication of the gavage procedure is the perforation of the esophagus or stomach (Shimizu *et al.*, 2004; Bonnichsen *et al.*, 2005). Additional complications involve the accidental introduction of fluids into the trachea or lungs, asphyxia, inflammation, weight loss, hemorrhage, and reflux (Bonnichsen *et al.*, 2005; Damsch *et al.*, 2011). Numerous factors can influence the success of gavage, including the level of experience of the technician, size and type of probe used, the volume administered, repetitive dosing and the vehicle used (Kai *et al.*, 2006). Yet, histological analysis confirmed there were no morphological changes in the lungs nor was the weight of the lungs significantly different between groups, suggesting intragastric administration was successfully performed.

5.5 Nociceptive behaviuor: mechanical hyperalgesia

The major challenge of animal experiments in pain is the absence of verbal communication (Mogil, 2009). Non-human animals cannot self-report, but their behaviours in response to noxious stimuli can be reliably and objectively scored (Blackburn-Munro, 2004; Mogil, 2009).

Pain originating from muscle or joint is uniquely different from pain originating from skin. Muscle pain is diffuse, longer lasting and more unpleasant. Patients with arthritic joints usually report mechanical hyperalgesia (Radhakrishnan *et al.*, 2003; Imamura *et al.*, 2008). Furthermore, neuropsychiatric disorders represent a leading cause to develop chronic widespread pain. Patients with depression for instance, often complain about chronic pain (predominantly in the musculoskeletal system), which in some cases is treatable with antidepressant drugs (Lomazzo *et al.*, 2015).

The K/C model is known to induce mechanical hyperalgesia (Amorim *et al.*, 2014) and is used for the evaluation of potential antinociceptive drugs (Lawand *et al.*, 2000). In our study, mechanical stimulations of rats elicited behavioural responses that were clearly distinguishable from spontaneous body movements. Nociceptive behavioural responses were assessed at two different timepoints, before and after treatment, by applying pressure directly at the site of injury with the PAM device, since this method allows an accurate behavioral measurement of mechanical hypersensitivity in rodents with chronic inflammatory joint pain (Bagüés *et al.*, 2016). Our results

show ARTH animals exhibited mechanical hyperalgesia with responses decreased two-fold when compared to SHAM animals. After induction of the model an acute phase of inflammation was followed by a chronic phase, lasting up to 5 weeks. Changes in knee tissues after the initial insult are likely responsible for the sensitization of peripheral nociceptors and primary afferents and contribute to the development of secondary hyperalgesia.

The effects of the hydroalcoholic extract of Bd, during a five week daily treatment with 50mg/kg of Bd extract completely, and 100mg/kg partially, reversed mechanical hyperalgesia. Furthermore, our data suggests the involvement of the inflammatory pathways in this effect.

5.6 Emotional comorbidities

Mood disorders such as depression and anxiety are frequently observed in patients suffering from chronic pain (Yalcin *et al.*, 2011). Although this comorbidity is clinically well established, the underlying mechanisms remained unclear (Yalcin *et al.*, 2011). To date, animal studies investigating the relationships between neuropathic pain and mood disorders are limited and contradictory. Indeed, initial studies failed to show any association between neuropathic pain and anxiety- and depression-related behaviors (Kontinen *et al.*, 1999; Hasnie *et al.*, 2007) whereas other research groups observed anxiety and/or depression related phenotypes (Amorim *et al.*, 2014). However, the first studies were performed during the first 3 weeks following neuropathy induction, while others were performed at later time points, which is incoherent as time is needed for the development of emotional comorbidities in chronic pain models (Yalcin *et al.*, 2011). This suggests that the time factor may be critical to model the affective consequences of neuropathic pain and that the expression of the anxiety-like phenotype required four weeks and preceded the depressive-like behaviour which was only observed six to eight weeks post-induction (Yalcin *et al.*, 2011).

Taking this data into account, we extended the post-induction period to five weeks and demonstrated the development of both anxiety- and depressive-like behaviours.

5.6.1 Anxiety-like behavior

Our results show ARTH animals spend more time in the center of the arena, when compared to SHAM animals. Although they spend more time in the center, ARTH animals scored less in the number of entrances in the center of the arena. Moreover, the tendency to avoid the central area

is an indicator of anxiety-like levels, under the assumption that the central area is more threatening for rodents than the peripheral area (Prut & Belzung, 2003; Heredia *et al.*, 2014).

Our ARTH animals displayed a decreased of the exploratory behaviour, lower number of rearings, lower number of squares crossed, lower number of entrances in the central area and more immobility time, suggesting the development of an anxious-like phenotype (Prut & Belzung, 2003; Saenz *et al.*, 2006).

An increase in locomotion, in the time spent and in the number of vertical rearings in the central part of the arena can be interpreted as an anxiolytic-like effect while the opposite, a decrease in these variables, is associated with an anxiogenic effects (Prut & Belzung, 2003)

In comparison to ARTH animals, Bd treated animals display a partial reversal of the anxiety-like symptoms as these animals displayed increased exploreration activities (more rearings, more number of entrances in the central area, more number of squares crossed and less time of immobility). Our data is in accordance to those published by Reis *et al.* (2014) showing propolis administration, evaluated in the OFT, increased the percentage of central quadrants crossed, the percent of time spent in the central area and the number of grooming events, demonstrating anxiolytic-like effects at a dose of 50 mg/kg. *Baccharis dracunculifolia* is one of the main botanical sources of Brazilian propolis and these effects may be related to the composition of the plant extract.

Moreover, some natural flavonoids, such as apigenin, also have a selective and relatively mild affinity for benzodiazepine receptors and chlorogenic acid possesses dual effects – cytoprotective and anxiolytic - which may be beneficial to anxious subjects (Bouayed, 2010).

5.6.2 Depressive-like behavior

5.6.2.1 Learned Helplessness

The FST revealed significant differences between experimental groups (SHAM and ARTH), ARTH animals spent more time immobile and less time struggling and swimming. This type of behaviour is considered learned helplessness, which, presumably, represents human “resignation” (Amorim *et al.*, 2014).

Both doseges of the extract, were able to decrease immobility and to enhance active behaviors, simultaneously. ARTH animals treated with a dose of 100 mg/kg display a lower latency to

immobility, however, in the other evaluated parameters (Swimming, struggling and immobility), ARTH animals treated with a dose of 50 mg/Kg showed better results.

The noradrenergic and serotonergic systems are involved in different aspects of regulating cognitive-emotional functions. For example, the noradrenergic system is strongly involved in regulating vigilance, alertness, and motivation. In addition, the noradrenergic system also regulates several additional psychological functions, including sensory processing, synaptic plasticity, network tuning, and memory. By contrast, the serotonergic system is strongly involved in the regulation of emotional and behavioral control processes (Brühl *et al.*, 2011; Zhen *et al.*, 2012).

The FST is sensitive and relatively specific to all major classes of antidepressants including tricyclics, serotonin selective reuptake inhibitors, and MAO inhibitors (Emamghoreishi &Talebianpour, 2009; Brand & Harvey, 2016). Although all antidepressant drugs reduce immobility in the FST, two distinct active behavioral patterns are produced by pharmacologically selective antidepressant drugs, swimming is sensitive to serotonergic compounds, and climbing is sensitive to antidepressants and drug with selective effects on noradrenergic transmission (Brand & Harvey, 2016)

Our data showed treatment with 50mg/Kg of Bd extract increased the time spent in swimming and in struggling (diving and climbing) suggesting this Bd extract dose probably enhances norepinephrine and serotonin neurotransmission.

Almost every compound that inhibits monoamine reuptake, leading to an increased concentration of monoamines in the synaptic cleft, has been proven to be a clinically effective antidepressant (Hasler, 2010; Yan *et al.*, 2016). In order to verify whether the serotonergic and noradrenergic systems are involved in the antidepressant-like effects of Bd extract, it would be interesting to investigate changes in the levels of monoamines throughout the experimental period.

However, increased immobility in the FST could also be caused by spontaneous pain, and, consequently, less movement, independently of depression. We could not rule out this possibility, but we think it unlikely since for spontaneous pain to increase the immobility time, all ARTH rats would have had to experience spontaneous pain within the 5-min interval of the test, which is unlikely. Thus, we believe the increased immobility observed in the FST was a reflection of the development of depressive-like behavior.

5.6.2.2 Anedonic behaviour

Our ARTH animals showed a significant decrease in the preference for a sweet solution when compared to SHAM, hence displaying a clear hedonic deficit. After treatment with Bd extract, sucrose preference was significantly different between the experimental groups with Bd treated animals displaying more preference for the sugary beverage when compared to ARTH animals, especially at the dose of 100 mg/kg. This result was not expected since in the FST the best results were obtained with the lowest dose (50 mg/kg). Although the treatment of 100mg/kg had no effect upon learned helplessness, reversed the hedonic-like behaviour.

Recently, Reis *et al.*, (2014) reported propolis extract exerted antidepressant-like effects in the CNS in different animal models of chronic stress and Lee *et al.*, (2013) also showed antidepressant-like activity in mice submitted to stress, suggesting propolis could be used as an alternative treatment for patients with neuropsychiatric disorders and a novel therapy for depression.

Bd extract ameliorates the symptoms of depressive-like behaviours, like anhedonia and learned helplessness. Together our results suggest that the Bd extract could provide a protective function against the depressive symptoms of pain.

5.7 Activation of microglia in brain induced by pain and depression-like behavior

In the current study, we focused on microglial cells because mounting evidence shows activation of these cells contributes to the plasticity of synaptic transmission in chronic pain states (Thakur *et al.*, 2012; Malfait & Schnitzer, 2013; Tran *et al.*, 2017).

Moreover, recent studies suggest glial activation and neuroinflammation play an important role in the pathogenesis of psychiatric and neurodegenerative diseases. Activated glial cells secrete various cytokines that influence neurotransmission, hypothalamus– pituitary–adrenal axis activity, neuronal plasticity and neurogenesis. It has been suggested alterations in cytokine networks are also involved in the mechanism of action of antidepressant drugs (Bielecka et a., 2010). In addition to their classical effects on neurotransmission, antidepressant drugs can also inhibit the production of proinflammatory cytokines and suppress microglial activation (Yirmiya et a., 2015)

Blocking microglial activation alleviates pain hypersensitivity in several animal models of chronic pain, indicating the activation of microglia contributes to enhanced pain sensitivity. These studies

form an emerging perspective that recognizes brain microglia as a key mechanism in the progression of chronic pain phenotypes (Malfait & Schnitzer, 2013; Taylor *et al.*, 2016).

Several antidepressants (including TCAs, serotonin/norepinephrine reuptake inhibitors and monoamine oxidase inhibitors) were also found to have microglia-suppressive effects in microglia cultures (Hashioka *et al.*, 2007; Bielecka *et al.*, 2010) and long-term antidepressive treatments (with either imipramine or fluoxetine) prevented the activation of microglial cells *in vivo*, as well as development of depressive-like behavior (Obuchowicz *et al.*, 2014).

In this work, we used an immunohistochemical evaluation to examine the response of microglial cells to OA. We initially examined the number of IBA-1 + cells in order to determine the total number of activated microglial cells and verified an increase in the number of IBA-1 + microglia in all analyzed areas (CC, ILC, PLC, AMY, DLPAG and LPAG) in ARTH groups in comparison with the SHAM group. These results demonstrated OA induces a significant increase in microglial activation in the brain, an effect particularly exacerbated in the AMY. Oral administration of the Bd extract decreased the number of IBA-1 + microglia cells to basal values, similar to those found in the SHAM group.

By retracting their processes when highly activated, microglia cells occupy a smaller area in the brain parenchyma than when moderately activated, which leaves larger areas between cells.

As in other chronic pain models, we observed an interesting correlation between the development of microglial activation and pain-like behavior. Only recently have researchers started to explore microglial activation associated with experimental OA. Using the monoiodoacetate (MIA) model, in a rat study by Thakur *et al.* (2012) and in a mouse study by Ogbonna *et al.* (2012), the number of dorsal horn IBA1 + cells was enhanced in arthritic animals when compared with controls. Another study (Tran *et al.*, 2017) showed that after destabilization of the medial meniscus (DMM), an increase in the number of activated microglia in the dorsal horn was observed when compared to controls.

As *Baccharis dracunculifolia* is thought to produce anti-inflammatory effects it may modulate the microglial activation that occurs in response to chronic pain, mainly by antagonizing the activity of microglial cells. Our study suggests microglial modulators may be beneficial for chronic pain patients, and thus provides a rationale for exploring the role of microglia as therapeutic target for treating chronic pain.

However, in order to explain the role of the Bd extract in the inhibition of microgliosis and in morphological changes of microglia cells, it may be important to evaluate the pro-inflammatory cytokines produced by microglia cells that may be involved in the pathogenesis of depression. Additionally, we can evaluate the microgliosis in spinal dorn horn, to understand if Bd extract act as inhibitor at an early stage and if this may influence the microglia phenotype change.

6.1 Conclusion

In this study, Bd extracts demonstrated an antioxidant action that may be linked to its chemical composition, namely its content in flavonoids (Apigenin) and other phenolic compounds (Caffeoylquinic-acid).

We also showed K/C-induced OA leads to the development of anxiety-, and depressive-like behaviour in rats, an effect probably partly due to the activation of microglia in brain areas involved in the modulation of pain and emotions. Improvements in these parameters after treatment with Bd extract were paralleled by a decrease in the number of supraspinal activated microglial cells, highlighting Bd as a potential drug for the control of pain and associated mood disorders in OA. In addition, drugs targeting microglia activation might be suitable candidates for the development of novel pain management therapies.

6.2 Future perspectives

Additional studies can be performed in order to fully understand the effects of Bd extract, namely to:

- Isolate individual Bd compounds to understand which ones display greater anxiolytic, anti-depressive and anti-nociceptive potential.
- Test a dose of 25 mg/kg since in most parameters the dose of 50 mg/kg presents better results than the dose of 100 mg/kg.
- Evaluate neuronal changes throughout the development of the disease in order to understand in which week the microglia is activated and how the Bd extract decreases it.
- Try the encapsulation of Bd extract in liposomes as a potentially novel strategy to facilitate the delivery of polyphenols across the blood-brain barrier and also to effectively reduce the active dose (Bouayed, 2010).

7.1 References

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Annex 1

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

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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.

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

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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

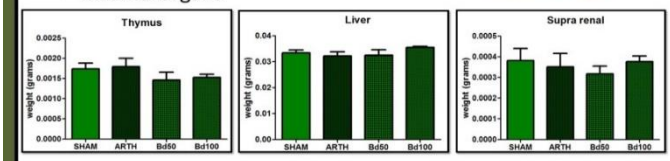
Materials and methods

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



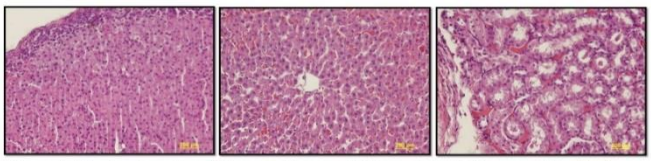
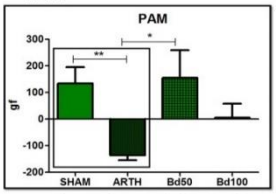


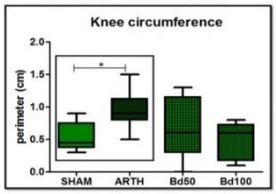
Figure 1. Photomicrophs of thymus, liver and kidney slides (400x magnification).

❖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

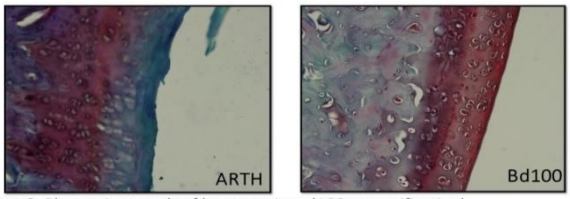
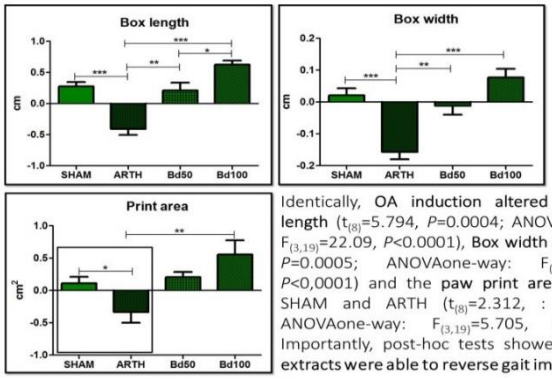


Figure 2. Photomicrograph of knee sections (100x magnification).

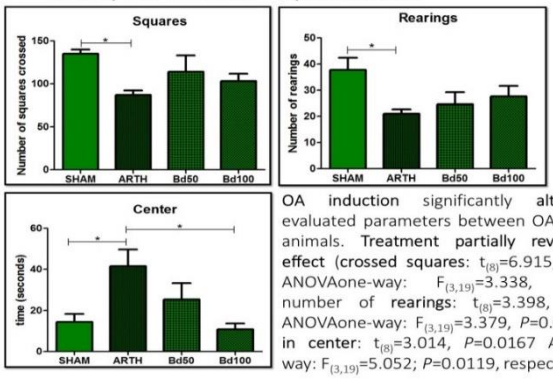
- Catwalk test - coordination and locomotor ability



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

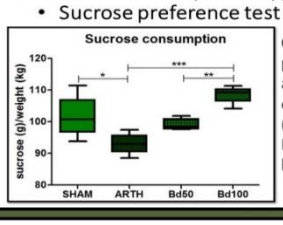
- Anxiety-like behaviour – Open-field test



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

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Annex 2:

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

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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.

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

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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.

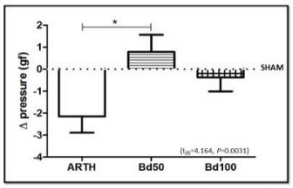
Methods

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.

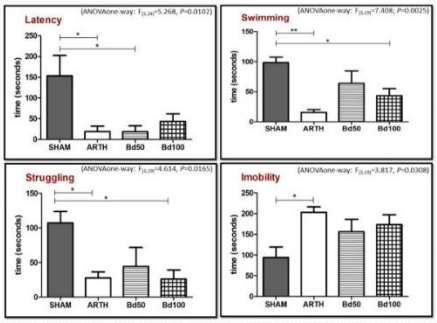
Results

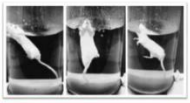
PAM



ARTH significantly **increased mechanical hyperalgesia**, an effect **reversed** by treatment with **Bd extract** (ANOVAoneway: $F_{(3,31)}=4.066$, $P=0.025$)

FORCED SWIMMING TEST



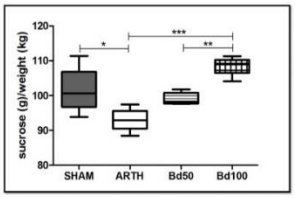


ARTH decreased:
- **latency** to immobility
- time spent **swimming**,
- time spend **struggling**

ARTH increased immobility

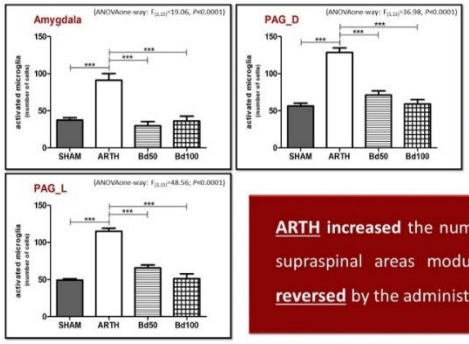
Bd extract partially reversed depression-like behaviour

SUCROSE PREFERENCE TEST



ARTH-induced **anhedonia** was **reversed** by treatment with **Bd extract** (ANOVAone-way: $F_{(3,31)}=13.62$; $P=0.0001$).

IMMUNOHISTOCHEMISTRY



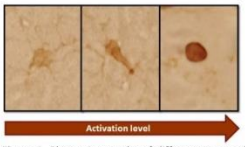


Figure 1. Photomicrographs of different stages of activated microglia (40x magnification).

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***

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