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JULLYANA DE SOUZA SIQUEIRA QUINTANS

EFEITO ANTINOCICEPTIVO DO ACETATO DE HECOGENINA EM CAMUNDONGOS – PARTICIPAÇÃO DO SISTEMA OPIÓIDE

ARACAJU
2013

QUINTANS/JULLYANA
DE SOUZA SIQUEIRA

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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Sergipe como requisito para obtenção do título de Doutor em Ciências da Saúde.

ORIENTADOR: Prof. Dr. Ângelo Roberto Antoniolli

CO-ORIENTADOR: Prof. Dr. Valter Joviniano Santana Filho

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PARECER

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RESUMO

QUINTANS, J.S.S. **EFEITO ANTINOCICEPTIVO DO ACETATO DE HECOGENINA EM CAMUNDONGOS – PARTICIPAÇÃO DO SISTEMA OPIÓIDE.** Tese de Doutorado em Ciências da Saúde, Universidade Federal de Sergipe, 2013.

Acetato de Hecogenina (HA) é uma sapogenina esteroidal acetilada, utilizada como um importante precursor pela indústria farmacêutica para a síntese de hormônios esteróides, tais como progesterona e prednisona. No entanto, existem poucos estudos farmacológicos sobre o HA e não foram encontrados estudos especificamente sobre o seu possível efeito analgésico. Desta forma, o objetivo deste estudo foi avaliar o perfil antinociceptivo do HA em camundongos, em modelos de nocicepção crônica e aguda. O pré-tratamento agudo por via intraperitoneal (i.p.) com HA (5 - 40 mg/kg) produziu um aumento dose dependente no tempo de latência ($p<0,01$) da retirada da cauda no teste do tail-flick em relação ao grupo tratado com o veículo, sugerindo um efeito analgésico central. Quando avaliada juntamente com antagonistas farmacológicos, o HA (40 mg/kg) teve seu efeito revertido pela naloxona (um antagonista não seletivo do receptor opióide, 5 mg/kg), CTOP (antagonista do receptor μ opióide, 1 mg/kg), nor-BNI (antagonista do receptor K opióide, 0,5 mg/kg), naltrindole (antagonista do receptor δ opióide, 3 mg/kg), ou glibenclamida (bloqueador do canal para K sensível ao ATP, 2 mg/kg), sugerindo um efeito sobre o sistema opióide. O efeito antinociceptivo do HA não parece estar relacionado a um possível perfil miorrelaxante, uma vez que camundongos tratados com HA (5 - 40 mg/kg) não apresentaram alterações na função motora quando avaliados no teste da coordenação motora. A administração sistêmica de HA (5 - 40 mg/kg) aumentou o número de células positivas para proteína Fos na substância cinzenta periaquedutal e, por outro lado, o pré-tratamento agudo com HA, em todas as doses testadas, inibiu significativamente a expressão de Fos no corno dorsal da medula, sugerindo um possível efeito sobre a via descendente de controle da dor. Adicionalmente, a administração i.p. de HA. (5, 10, ou 20 mg/kg), inibiu de maneira significativa ($p<0,05$ ou $p<0,001$) a hiperalgesia mecânica induzida pela carragenina, TNF-α, dopamina e PGE_2 em camundongos. Ao investigar os possíveis efeitos de HA (20 ou 40 mg/kg, i.p.) sobre a hipersensibilidade neuropática crônica (modelo de ligação parcial do nervo isquiático - PSNL), o tratamento agudo com HA foi eficaz em reduzir significativamente ($p<0,01$) o comportamento hiperalgésico, sem alterar os parâmetros motores dos animais. Diante desses resultados, o presente estudo sugere, pela primeira vez, que o HA possui perfil antinociceptivo consistente com mecanismos mediados pelo sistema opióide, e que este composto pode ser útil no estudo de novas abordagens terapêuticas para o tratamento farmacológico da dor.

Descritores: Acetato de Hecogenina, dor, proteína Fos, sistema opióide

ABSTRACT

SIQUEIRA, J. S. **ANTINOCICEPTIVE EFFECT OF HECOGENIN ACETATE IN MICE - PARTICIPATION OF THE OPIOID SYSTEM.** Tese de Doutorado em Ciências da Saúde, Universidade Federal de Sergipe, 2013.

Hecogenin acetate (HA) is a steroidal sapogenin-acetylated, one of the most important precursor used by the pharmaceutical industry for the synthesis of steroid hormones. However, no studies were found on the possible analgesic profile of HA. Thus, we aimed to evaluate antinociceptive profile of HA in chronic and acute animal models. Acute pretreatment with HA (5 – 40 mg/kg) produced a dose dependent increase in the tail flick latency time when compared to vehicle-treated group ($p<0.01$) demonstrating central analgesic effect. The antinociceptive effect of HA (40 mg/kg) was prevented by naloxone (a non selective opioid receptor antagonist; 5 mg/kg), CTOP (μ opioid receptor antagonist; 1 mg/kg), nor- BNI (k opioid receptor antagonist; 0.5 mg/kg), naltrindole (δ opioid receptor antagonist; 3 mg/kg), or glibenclamide (ATP sensitive K^+ channel blocker; 2 mg/kg). This effect no seems to be related to a possible myorelaxing profile of HA, since mice treated with HA (5 - 40 mg/kg) did not show motor performance alterations. Systemic administration of HA (5 - 40 mg/kg), increased the number of Fos positive cells in the periaqueductal gray and the acute pretreatment with HA, at all doses tested, significantly inhibited the Fos expression in the spinal cord dorsal horn. Additionally, intraperitoneal administration of HA (5, 10, or 20 mg/kg; i.p.) inhibited the development of mechanical hyperalgesia induced by carrageenan, TNF-α, dopamine and PGE_2 in mice. When we investigated effects of HA (20 or 40 mg/kg, i.p.) on chronic neuropathic hypersensitivity (partial sciatic nerve ligation - PSNL), the acute treatment with HA was effective in producing a significanty ($p<0.01$) anti-hyperalgesic effect in PSNL model on mice. So, the present study demonstrates, for the first time, that HA produced consistent antinociception mediated by opioid receptors and endogenous analgesic mechanisms and that this compound may be useful in the study of new therapeutic approaches to pain treatment.

Keywords: Fos protein, Hecogenin acetate, Opioid system, Pain

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INTRODUÇÃO

1. INTRODUÇÃO

A dor está ligada ao homem desde os primórdios da humanidade. É considerada um feno□meno complexo, multifacetário, que envolve aspectos afetivo-motivacionais, sensitivo-discriminativos e cognitivo-avaliativos sendo um dos sintomas mais frequentes no contexto das doenc□as (Pimenta et al., 1997, Calvino e Grilo 2006). Apesar da atual compreensão da neurofisiologia da dor estar em franco processo de amadurecimento pela medicina, de acordo com Hargreaves (2011), o tratamento adequado da dor continua sendo o maior desafio da medicina moderna, devido a fatores como o limitado arsenal terapêutico para tipos específicos de dores, número considerado consistente de reações adversas produzido pelos analgésicos disponíveis, bem como o uso inapropriado das opções terapêuticas existentes.

Na verdade, apesar dos avanços farmacológicos recentes na farmacoterapia, os fármacos opióides e os anti-inflamatórios não esteroidais (AINEs) continuam sendo os fármacos mais importantes no tratamento das condições dolorosas (Jaggi et al., 2011). De acordo com Kissin (2010), entre os anos de 1960 e 2009 foram aprovados pelo Food and Drug Administration (FDA), 59 novas entidades químicas utilizadas para o tratamento da dor, sendo que destas, 39 foram especificamente desenvolvidas como analgésicos. Nesse mesmo trabalho, Kissin ressalta que a busca por novos analgésicos, mais eficazes e/ou que apresentem menores reações adversas, se faz urgente principalmente para o tratamento de dores crônicas, tais como as neuropatias e a fibromialgia.

Entretanto, em estudo realizado por Li e Vederas (2009), estes sugerem uma crise no desenvolvimento de novas moléculas para fins farmacológicos pelo setor farmacêutico, mas destacam que apesar da sistemática diminuição do número de novas entidades químicas, cerca de 40 % dos novos fármacos continua sendo obtidos a partir dos produtos naturais. De fato, o último grande lançamento inovador do setor farmacêutico para o tratamento da dor foi o Ziconotide (Prialt®), inicialmente extraído do molusco Conus magus, que produz suas propriedades farmacológicas através do bloqueio de canais para Ca^{+2} ativados por voltagem (Li e Vederas, 2009). Nesse sentido, Barreiro e Bolzani (2009) destacam a importância dos produtos naturais como fonte de

novas moléculas que atualmente não podem ser sintetizadas ou ainda como substâncias que possuem uma arquitetura molecular inovadora.

Dentre os produtos naturais com notória importância para o setor farmacêutico tem se destacado as sapogeninas esteroidais, tais como diosgenina e hecogenina, presentes em várias espécies vegetais do gênero Agave (conhecido popularmente como "sisal"). Estas substancias tem sido amplamente utilizadas pela indústria farmacêutica como moléculas precursoras na síntese de hormônios esteroidais, tais como os adrenocorticóides e hormônios correlatos (cortisol, dexametasona, cortisona, prednisona, etc.), hormônios sexuais (progesterona) e anabolizantes (estanozolol, metandienona) (Hostettmann e Marston, 1995; Sakai e Koezuka, 1999; Ghoghari e Rajani, 2006).

Alguns trabalhos tem demonstrado que a hecogenina (Figura 1A) possui importantes propriedades biológicas (Peana et al., 1997; Brito et al., 2011; Cerqueira et al., 2012; Fernández-Herrera et al., 2012). Dentre as propriedades farmacológicas atribuídas a essa sapogenina destaca-se seu efeito anti-inflamatório e anti-edematogênico sugerindo que essas propriedades podem estar relacionados com sua ação sobre canais para K^+ sensíveis ao ATP além do seu pronunciado perfil antioxidante (Corbiere, 2003; Peana et al., 1997; Cerqueira et al., 2012). Recentemente, Cerqueira et al. (2012) demonstraram que a hecogenina apresentou efeito anti-inflamatório e gastroprotetor através da expressão da cicloxigenase-2 (COX-2) e pela redução da enzima mieloperoxidase.

Por outro lado, a hecogenina, como outras sapogeninas obtidas do "sisal", são relativamente instáveis no meio ambiente, podendo hidrolisar com facilidade (Silveira et al., 2012). Desta forma, a inclusão do grupo acetato tem tornado a hecogenina quimicamente mais estável, contudo, podendo alterar suas propriedades farmacológicas. De fato, não há relatos encontrados na literatura especializada com estudos descrevendo as propriedades farmacológicas do acetato de hecogenina (Figura 1B), uma sapogenina esteroidal acetilada. Apesar disso, existem importantes exemplos descritos na literatura onde a inclusão deste ânion orgânico ($CH3COO^-$) contribuiu para melhorar a eficácia do efeito anti-inflamatório (no caso do ácido acetilsalicílico) ou para reduzir a toxicidade (acetato de hidrocortisona) de fármacos amplamente utilizados na clínica médica (Barreiro e Bolzani, 2009).

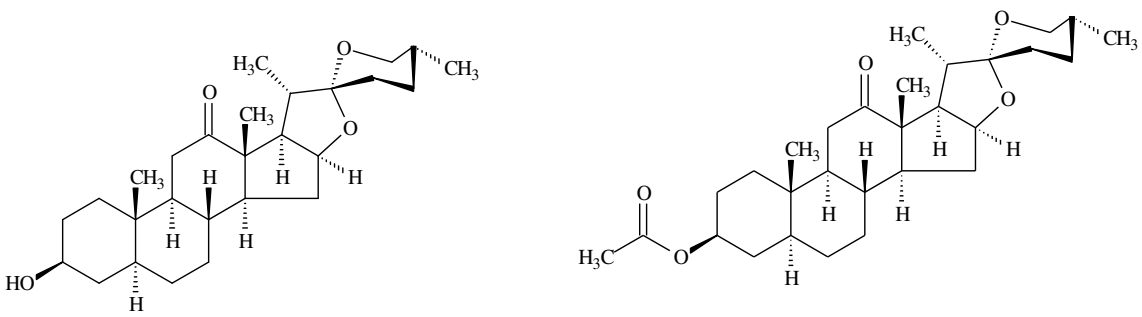

Figura 1 – Estruturas moleculares planas da hecogenina **(A)** e do acetato de hecogenina **(B)**

Trabalhos recentes tem descrito que os compostos esteróides, notoriamente utilizados na clínica no controle de vários processos inflamatórios, podem ser promissores no manejo de dores agudas e crônicas (Jevtovic-Todorovic et al., 2009; Strong et al., 2013). Os neuroesteróides são importantes na modulação de uma variedade de funções sobre o Sistema Nervoso Central (SNC), inclusive como entidades químicas promissoras para o tratamento das dores neuropáticas e inflamatórias (Mensah-Nyagan et al., 1999; Melcangi et al., 2008). Yarushkina et al. (2011) e Wand et al. (2012) têm chamado a atenção para a correlação entre compostos esteroidais e o sistema opióide buscando melhorar os efeitos farmacológicos e reduzir as já bem conhecidas reações adversas de ambos.

Desta forma, o presente estudo buscou contribuir com o conhecimento farmacológico desta sapogenina esteroidal acetilada avaliando sua atividade em modelos de nocicepção aguda, inflamatória e neuropática em roedores, buscando caracterizar seu possível envolvimento em áreas do SNC.

REVISÃO DA LITERATURA

2. REVISÃO DA LITERATURA

2.1 Natural products evaluated in neuropathic pain models – a systematic review

Artigo a ser submetido à Phytomedice (Fator de Impacto-JCR 2011: 2,972)

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Abstract

Chronic pain conditions, such as neuropathic pain, are a common problem that poses a major challenge to health-care providers due to its complex natural history, unclear etiology and poor response towards therapy. Despite the large number of drugs available, the adherence is limited by the large range of side effects and pharmacological ineffectiveness. Thus, the search for new chemical entities that can act as promising molecules to treat chronic has emerged. The natural products remain as the most promising sources of new chemical entities with applicability for the medical approach. Henceforth, we performed a systematic review analyzing pre-clinical studies shown to be promising in a possible applicability in neuropathic pain. The search terms neuropathic pain, phytotherapy, and medicinal plants were used to retrieve English language articles in LILACS, PUBMED and EMBASE published until March 2013. From a total of 1,539 articles surveyed, 28 met the inclusion and exclusion criteria established. The main chemical compounds studied were flavonoids (32%), terpenes (18%) and alkaloids (14%). The mostly described animal models for the study of neuropathic pain included were Chronic constriction injury (CCI - 32%), Partial Sciatic Nerve Ligation (PSNL - 28%), Streptozotocin - induced diabetic (28%), alcoholic neuropathy (3.5%), sodium monoiodoacetate (MIA - 3.5%), and neuropathic pain induced by paclitaxel (3.5%). The opioids, serotonergic and cannabinoid systems are suggested as the most promising targets for the natural products described. Therefore, the data reviewed here suggest that these compounds are possible candidates for the treatment of chronic painful conditions, such as neuropathic pain.

Keywords: Medicinal plants, natural products, neurophatic pain, pain

INTRODUCTION

Pain is one of the most important health problems worldwide and it remains as an important challenge of modern medicine (Nguelefack et al., 2010). Chronic pain is a public health problem that causes personal and social afflictions. Depending on its origin, chronic pain can be classified as inflammatory or neuropathic. Neuropathic pain can arise from a disease or injury to the central nervous system (CNS) or the peripheral nervous system (PNS). Millions of people are suffering from this chronic condition worldwide that could comprise their engaging in daily activities (Hall et al., 2006; Amin and Hosseinzadeh, 2012).

Several pathologies result from overt or silent injuries affecting secondarily the CNS injury or, more commonly, in association with an injury to the PNS such as amputation pains, complex regional syndromes, radiculopathies, herpes zoster and diabetic neuropathies. These injuries can be caused by tumors compressing peripheral nerves, the toxins used in chemotherapy, metabolic disease, viral disease, severe ischemic insults, trauma and disc herniations that stretch, compress or inflame a nerve root (Saadé and Jabbur, 2008; Valsecchi et al., 2008).

The neuropathic pain is usually difficult to treat because the etiology is heterogeneous and the underlying pathophysiology is complex (Woolf and Mannion, 1999; Zimmermann, 2001; Amin and Hosseinzadeh, 2012). The available drugs have often limited therapeutic potential in the management of the chronic pain. Moreover, they often put patients at risk due to their common side effects (Woolf and Mannion, 1999). Then neuropathies continue to pose challenges towards both medical treatment and scientific research (Saadé and Jabbur, 2008).

Because there is no universally efficacious therapy for it, neuropathic pain research has been explored with different animal models where intentional damage is

inflicted to the sciatic nerve, branches of spinal nerves or in the spinal cord (Zimmermann, 2001; Ji and Stricharstz, 2004). Although these models are not perfect, the development of such experimental models is essential, not only for the detection of new analgesics, but also for a better understanding of pain syndromes that are difficult to manage clinically (Besson, 1999).

Despite the great synthetic diversity derived from the development of combinatorial chemistries and high-throughput screening methods over the past fifty years, natural products and related structures continue to be extremely important elements of pharmacopoeias (Ngo et al., 2013). In this context, natural products (NPs) or secondary metabolites which present fewer side effects emerge as interesting therapeutic resources for the development of new drugs for the management of certain chronic pain states. In fact, NPs traditionally have played an important role in drug discovery and were the basis of most early medicines (Butler, 2008; Li and Vederas, 2009).

Despite its importance, there are no systematic reviews on the analgesic potential of NPs for neuropathic pain. Accordingly, we conducted a systematic review of the literature to examine and synthesize the literature on NPs and then identify those that assess antinociceptive effects in neuropathic pain models.

METHODS

This systematic review was conducted in accordance with the guidelines of Transparent Reporting of Systematic Reviews and Meta-Analyses (PRISMA statement).

Search Strategy

Three internet sources were used to search for appropriate papers that fulfilled the study purpose. These included the National Library of Medicine, Washington, D.C. (MEDLINE-PubMed), EMBASE (Excerpta Medical Database by Elsevier) and LILACS (Latin American and Caribbean Health Sciences), using different combinations of the following keywords: Neurophatic pain, Phytotherapy, Natural Products, Medicinal Plants. The databases were searched for studies conducted in the period up to and including April 10, 2013. The structured search strategy was designed to include any published paper that evaluated the use of natural products in neurophatic pain to identify those that show therapeutic potential. Citations were limited to animal studies. Additional papers were included in our study after the analyses of all references from the selected articles. We did not contact investigators and did not attempt to identify unpublished data.

Study Selection

All electronic search titles, selected abstracts and full-text articles were independently reviewed by a minimum of two reviewers (J.S.S.Q. and J.R.G.S.A.). Disagreements on study inclusion/exclusion were resolved with a consensus reaching. The following inclusion criteria were applied: neurophatic studies and the use of compounds obtained from medicinal plants for treatment. Studies were excluded according to the following exclusion criteria: studies in humans, studies using extracts or mixtures (as essential oils), review articles, meta-analyses, abstracts, conference proceedings, editorials/letters and case reports (Fig1).

INSERT FIGURE 1

Data Extraction

Data were extracted by one reviewer using standardized forms and were checked for completeness and accuracy by a second reviewer. Extracted information included data regarding the substance, animals models, dose, route and pharmacological mechanism of action suggested.

Results

A total of 1,539 abstracts/citations were identified from electronic and hand searches for preliminary review. After removal of duplicates and screening for relevant titles and abstracts, a total of 44 articles were submitted for a full-text review. Twenty eight articles fulfilled the inclusion and exclusion criteria established.

From 28 final selected studies (Figure 1), most of those researches were conducted in India (32%), Brazil (25%) and China (18%) (Table 1). Taking into consideration the isolated compounds (Figure 2), studies selected demonstrated 26 compounds that can be chemically classificated as flavonoids (28%), terpenoids (17%), alkaloids (14%), phenols (10%) and carotenoids (10%). In most articles analyzed, the substances were purchased commercially (60.7%). Only 12 studies were conducted as compounds isolated from plants (39.2%).

The animal models used to study neurophatic pain include PSNL, Streptozotocin-induced diabetes, CCI, alcoholic neuropathy, MIA and neuropathic pain induced by paclitaxel. However the main models used were CCI (32%), Streptozotocin-induced diabetes (28%) and PSNL (28%).

Regarding the mechanisms of action in the neuropathic pain proposed for the various substances, there were citations on the inhibition of protein kinase C,

antioxidant activity, antinflamatory action (inhibition of cytokines and expression of NF-kB), involvement of the opioid and dopaminergic systems and nitric oxide pathway, activation of cannabinoid receptors and interaction with both TRPV1 and TRPA1 receptors.

Discussion

The use of natural products, mainly medicinal plants, for medicines seeking treatment, cure and prevention of diseases, is one of the oldest forms of medical practice in the humanity. Recent studies reinforce the importance of natural products as a source of drugs (Barreiro and Manssour, 2008, Li and Vederas, 2009). Additionaly, herbal medicines and related compounds are reported to be beneficial in the management of painful neuropathy (Muthuraman and Singh, 2011; Garg and Adams, 2012). Following this current, we focused on the review of substances isolated from medicinal plant, since these also provide structural templates to obtain a synthetic compound. Also, they may be employed as a tool in identifying mechanisms of action (McKeena, 1996).

India and China are two of the largest countries in Asia, which have the richest arrays of registered and relatively well-known medicinal plants (Raven, 1998; Kala et al., 2006). Recent studies indicate that the majority of the Indian plant species demonstrates medicinal applicability and their use in ancient medicine, as Ayurvedic medicine, has confirmed their therapeutic profiles (Staud, 2011; Roeder and Wiedenfeld, 2013). Thus, in some of the Indian regions, over 50% of the plant species are regarded as medicinal plants and are widely used by the population to treat a large number of diseases (Rajeswara et al., 2012). In China, about 40% of the total medicinal consumption is attributed to traditional tribal medicines (Hoareau and DaSilva, 1999).

Although Brazil does not have a traditional medicine which is so famous as is that found in India and China, the use of natural products was among native peoples (indigenous) at least two thousand years before the discovery of the country by Portugal. Nowadays, the use of natural products has grown in Brazil, as well as the use of herbal medicine and related therapies (Brandão et al., 2008). The high prevalence of studies from India, Brazil and China demonstrated in our review probably occurred due to the vast biodiversity ('mega-biodiversity') found in these countries and the already-cultural use of medicinal plants or related natural products to treat several types of ailments (Ngo et al., 2013)

These naturally occurring secondary metabolites, most bearing complicated architectural skeletons, often exhibit significant and diverse biological activities. Accordingly, the development of therapeutic agents based on NPs represents a promising aspect in new drug discovery. Regarding secondary metabolites studied in this review, flavonoids are a group of naturally occurring polyphenolic compounds found ubiquitously in the plant kingdom (Andersen, 2005; Grotewold, 2006). Besides their relevance in plants, it has been shown that flavonoids are pharmacologically active in humans (Havsteen, 2002).

Flavonoids are one of the most important secondary metabolites for pharmaceutical and cosmetics industries due to their biological properties, such as cancer-preventive component, anti-aging, antioxidant, anti-inflammatory and analgesic (Shahidi, 1992; Niiveldt et al., 2001; Le Marchand, 2002). In fact, flavonoids exhibit a wide range of biological activities with different therapeutic applications, which are mainly attributed to their powerful antioxidant activity (Shahidi, 1992; Pietta, 2000) and/or modulation of enzymatic activities (Middleton and Kandaswami, 1994; Bors et

al., 1996). In our review, this family of compounds can manage pain mainly through its antioxidant activity and by modulating the activity of protein kinase C.

Our results demonstrated that flavonoid compounds have been used in the highest percentage in the streptozotocin (STZ)-induced diabetes model. This model mimics the diabetic neuropathy which is one of the most frequent peripheral neuropathies associated with hyperalgesia, cold allodynia and hyperesthesia, in which high glucose-induced oxidative and nitrosative stress have been proposed to be an important mechanism of neuronal injury relevant to diabetic neuropathy (Mabley and Soriano, 2005). Oxidative-nitrosative stress is an important determinant of degenerative and painful pathological conditions in peripheral nerve fibers (Chung, 2004; Drel et al., 2007; Vincent et al., 2002).

Reactive oxygen species sensitize nociceptors so that they not only respond more vigorously towards noxious stimuli, but also start to respond towards normally sub-threshold stimuli. This peripheral sensitization not only induces pain directly, but also induces central sensitization in the spinal cord, which indirectly contributes to pain as well (Wang, 2004). Superoxide accumulates and high concentrations combine with nitric oxide to form peroxynitrite, which is implicated in diabetes-associated motor and sensory nerve conduction deficits, as well as peripheral nerve energy deficiency (Drel et al., 2007; Kim et al., 2003).

The terpenoids form a large family derived from isoprene units. They are classified depending on the number of isoprene units as hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes and tetraterpenes. The terpenes are structurally simple molecules, but reported studies on their pharmacological properties show their potential clinical use as analgesic drugs. They are all molecules with low molecular weight, usually with high lipid solubility. They can penetrate the blood-brain

barrier and act in the central nervous system (CNS). Therefore, they may present a profile of psychoactive drugs (De Sousa, 2011). Moreover, Guimarães et al (2013), in a recent systematic review, has described that terpenes can act in CNS modulating important neurotransmitter systems, such as glutamatergic, serotoninergic, opioid and cannabiboid, which have an important role in modulating chronic pain, such as neurophatic pain.

In this review, the 5 compounds classified as terpenes were tested in the model of neuropathic pain induced by partial ligation of the sciatic nerve (Seltzer et al., 1990). Experimental models of neuropathy produced by sciatic nerve injury in animals mimic symptoms observed in humans with nerve injury, and these models are extensively used in behavioral research (Bennet and Xie, 1988). In this experimental model, we can observe prolonged changes in neurotransmitter and receptor expression, producing central sensitisation in response towards the release of several inflammatory and pain mediators, which in turn increase the sensitivity of peripheral sensory afferents at the site of injury and in the central nervous system (Zimmermann, 2001; Basbaum, 1999; Petersen and Basbaum, 1999; Urban and Gebhart, 1999).

Most terpenes (3/5) in this review showed analgesic action by activating cannabinoid receptors CB1 and/or CB2. The substances isolated from species Cannabis sativa L. are called phenolic terpene (Mechoulam, 1973; Gertsch, 2008). However, in recent years, a number of substances isolated from plants other than Cannabis sativa have also demonstrated affinity for cannabinoid receptors, being then defined as phytocannabinoids. These compounds have anti-inflammatory effects by inhibiting mRNA expression of the cytokine tumor necrosis factor-alpha (TNF-α) in human monocytes/macrophages via activation of the CB2 receptor (Raduner et al., 2006; 2007).

Another phytocannabinoid described as selective agonist of the CB2 receptor is β-caryophyllene bicyclic sesquiterpene (Gertsch, 2008). The peripheral mechanism through which BCP produces anti-allodynic effect in the present findings is unclear. Therefore, it seems that the activation of peripheral CB2 receptors might decrease the sensitivity of primary afferent neurons by inhibiting the release of sensitizing substances from neighboring mast and immune cells. However, there are various lines of evidence whereby selective activation of peripheral CB2 receptors is sufficient to display antinociception in models of acute, inflammatory and nerve injury-induced nociception (Malan et al., 2001; Ibrahim et al., 2006; Hanus et al., 1999; Quartilho et al., 2003; Clayton et al., 2002; Ibrahim et al., 2003; Nackley et al., 2003).

Another class of chemical compounds often quoted in our review is the class of alkaloids. Alkaloids are low-molecular-weight, nitrogen-containing substances with characteristic toxicity and pharmacological activity. These properties, which have traditionally been exploited by humans for hunting, execution and warfare, have also been used for the treatment of disease (Mann, 1992). Different alkaloids have been described, indicating their structural and biosynthetic diversity compared to that of other secondary metabolites (Wink, 1999).

Many alkaloids have properties in the central nervous system, especially those derived from indole alkaloids. To compare the structures of gelsenicine and koumine, Xu and colleagues (2012) found that other than the indole ring, their molecular formula were quite different. Thus, they hypothesized that the indole ring may be the requisite for analgesia and that perhaps some specified stereo-conformations may be needed. It will be important for future studies to determine the structure-activity relationship between the G. elegans alkaloids.

Most of the pharmacological mechanisms proposed in the studies surveyed highlight four major therapeutic targets: pro- and anti-oxidant mechanisms, reduction of pro-inflammatory mediators (i.e. cytokines, NO, COX), modulation of classics neural systems involved in pain management (dopaminergic and mainly opioid) and promising system (cannabinoid, oxidonitrergic system and ion channels). In this regard, Kissin (2010) conducted an extensive survey of the analgesic drugs developed and approved by the FDA during the period of 1960 to 2009, and in this review Kissin highlights that the new drugs should look as new targets: cannabinoid, NMDA antagonists, N-type calcium channel blockers and TRPV1 agonists. Hence, our results suggest that natural product remain promising in the search for new analgesics and also that can act in pharmacological sites considered promising.

The urgent need to find an alternative medicine which can attenuate neuropathic pain effectively led researchers to find a drug from natural sources. This review sheds some secondary metabolites on the medicinal plants from different parts of the world, which have potent active ingredients to cure this painful disorder of the nervous system. The main objective of this review, besides the pharmacological characterization of the compounds, is to undoubtedly show new options for the treatment of pain. The evidence can be classified as encouraging for its relief in neuropathic and also because it warrants further study to determine the structure-activity relationship in secondary metabolites.

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Figure and Table

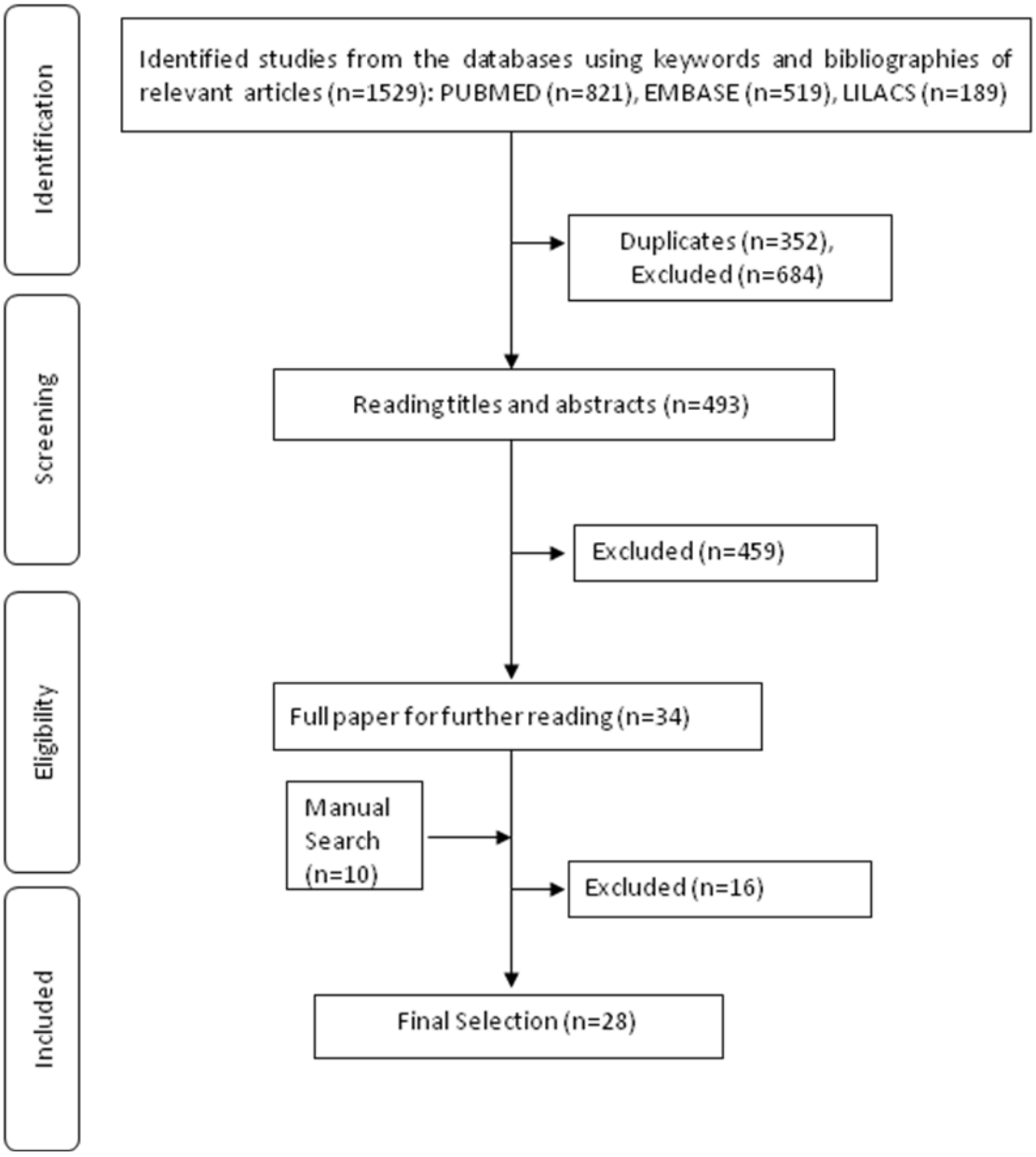


Figure 1. Search and selection results

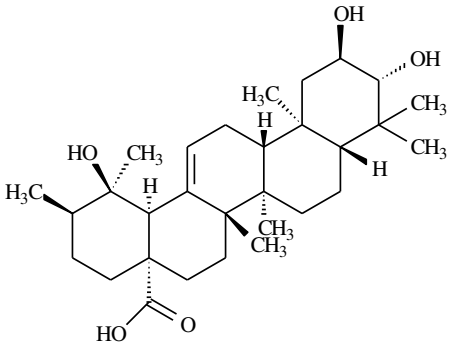
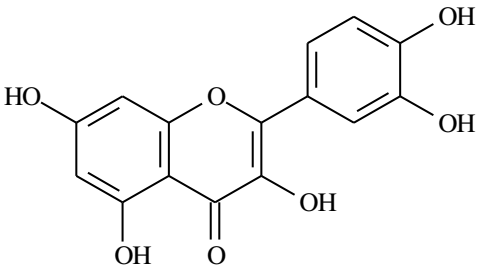
Table 1 – Characteristics of Included Studies

Authors, year, Country	Substance	Animals	Dose mg/kg (route)	Model	Mechanism of action
Bortalanza et al., 2002, Brazil	Tormentic acid **(1)**/ triterpene (V. divergens)	Male and female Swiss mice	30 mg/kg (p.o.)	PSNL	Antinociception was dissociated from its possible anti-inflammatory activity. Suggests that antinociception might be associated with ability to interact with PKC -dependent mechanisms.
Anjaneyulu & Chopra, 2003, India	Quercetin **(2)**/ flavonoid (Sigma)	Male albino mice of Laka strain	50, 100 mg/kg (p.o.)	STZ - induced diabetic	Antinociception either acts as a direct opioid receptor agonist or it causes the release of endo-opioids. Activity may involve inhibition PKC
Meotti et al., 2006, Brazil	Myricitrin **(3)**/ flavonoid (genus Eugenia)	Adult female Swiss mice	30 mg/kg (i.p.)	PSNL	Antinociception appear to occur through molecular mechanisms including inhibition of PKC and NO cell signaling, Ca 2+ and K + transport. Antioxidant activity
Anjaneyulu & Chopra, 2004, India	Quercetin **(2)**/ flavonoid (Sigma)	Male SD rats	10 mg/kg (p.o.)	STZ - induced diabetic	Improved neuronal blood flow through reactive oxygen species scavenging or direct vasorelaxant properties
Sharma et al., 2006, India	Resveratrol **(4)**/ stilbene (Sigma)	Male SD rats	10 mg/kg (p.o.)	STZ - induced diabetic	Scavenging activity on reactive oxygen species or by its direct vasorelaxant properties
Sharma et al., 2006, India	Curcumin **(5)**/phenolic compound (Sigma)	Male albino mice of Laka strain	15, 30 and 60 mg/kg (p.o.)	STZ - induced diabetic	Antinociception possibly through its inhibitory action on NO and TNF-α release
Costa et al., 2006, Italy	Cannabidiol **(6)**/ cannabinoid (GW Pharmaceuti cals)	Male Wistar rats	2.5, 5, 10 and 20 mg/ kg (p.o.)	CCI	Antinociception was associated with a reduction in the content of several mediators, such as prostaglandin E_2 (PGE_2), lipid peroxide and nitric oxide (NO), and in the over-activity of glutathione-related enzymes
Sharma et al., 2007, India	Resveratrol **(4)**/ stilbene (Sigma)	Male albino mice of Laka strain	5, 10 and 20 mg/kg (p.o.)	STZ - induced diabetic	Antinociception seem to result from its inhibitory effect on NO, TNF- α production and possibly reactive oxygen species
Valsecchi et al., 2008, Italy	Genistein **(7)**/ isoflavone (Sigma)	C57BL/6J male mice	1, 3, 7.5, 15, and 30 mg/kg (s.c.)	CCI	Immunomodulatory and anti-inflammatory activities (reduced peripheral and central nuclear factor- κB, nitric oxide system and pro-inflammatory cytokine over-activation).
Kuhad et al., 2008, India	Lycopene **(8)**/ carotenoid (Sigma)	Male albino mice of Laka strain	1, 2 and 4 mg/kg (p.o.)	STZ - induced diabetic	Antinociception possibly through its inhibitory action on NO and TNF-a release
Kuhad & Chopra, 2008, India	Lycopene **(8)**/carotenoid (Sigma)	Male Wistar rats	4 mg/kg (p.o.)	STZ - induced diabetic	Antinociception may be attributed a powerful antioxidant activity. However, may have improved neuronal blood flow by its direct vasorelaxant properties

Batista et al., 2010, Brazil	(-)-Linalool **(9)**/ monoterpene (Sigma)	Adult female Swiss mice	50 or 200 mg/kg, (i.p.)	PSNL	Antinociception, at least in part, by inhibit pro-inflammatory cytokines and modulate the NMDA-glutamatergic receptor
Hagenacker et al., 2010, Germany	Myricetin **(10)**/flavonoid (Sigma)	Adult male Wistar rats	0.1, 1 and 10 mg/kg (i.p.)	SNL	Antinociception may be related to its PKC-induced decrease of $I_{Ca(V)}$ in DRG neurons.
Silva et al., 2011, Brazil	α-amyrin **(11)** β-amyrin **(12)**/ triterpenes (Protium kleinii)	Male Swiss mice or male Wistar rats	3 - 30 mg/kg (p.o.)	PSNL	Activation of cannabinoid receptors and by inhibiting the production of cytokines and expression of NF- κB, CREB and cyclooxygenase 2.
Tiwari et al., 2011, India	Epigallocatechin-3-gallate **(13)**/polyphenol (DSM Nutritional Products)	Adult male Wistar rats	25, 50, 100 mg/kg (p.o.)	Alcoholic neuropathy	Potent anti-oxidant activity and modulation of inflammatory cascade
Isacchi et al., 2011a, Italy	Verbascoside **(14)**/phenylpropanoid glycoside (Lippia citriodora)	Male SD albino rats	10 - 600 mg/kg p.o. i.p.	CCI MIA	Analgesic properties can be excluded. Not require the opioid system to reverse hyperalgesia
Isacchi et al., 2011b, Italy	Salvianolic acid B **(15)** phenolic compound (S. miltiorrhiza)	Male SD albino rats	10 ml/kg (i.p.)	CCI	Not registered
Liu et al., 2011, China	Gelsenicine **(16)**/alkaloid (Gelsemium elegans)	Male ICR mice	0.8, 4, 20µg/kg (s.c.)	CCI	Enhances myelin repair indirectly by inhibiting the inflammatory response that contributes to fiber damage. Promote remyelination through modulation of neurotrophic factors. The effect on pain-related behavior could also be mediated by down-regulation of TNF- α
Ou et al., 2011, China	Lappaconitine **(17)**/ alkaloid (NICPB Products, China)	Adult male SD rats	4 mg/kg (i.p.)	CCI	Antinociception involves decrease of expression and sensitization of the P2X3 receptors of the rat DRG neurons
Silva et al., 2012, Brazil	(-)-cassine **(18)**/ alkaloid (Senna spectabilis)	Male Swiss mice	3, 30 or 60 mg/kg (p.o)	PSNL	Antinociception by interact with both TRPV1 and TRPA1 receptors and by inhibiting the up-regulation of cyclooxigenase-2 as well as inhibiting the phosphorylation of MAPK/ERK and the transcription factor NF- kB.
Xu et al., 2012a, China	Puerarin **(19)**/flavonoid (Kangenbei Pharmaceutical Limited Corporation)	Male SD rats	100 mg/kg/day (i.p.)	CCI	Antinociception mediated by $P2X_3$ receptors in dorsal root ganglion neurons.

Dutra et al., 2012, Brazil	Euphol **(20)**/ triterpene (Euphorbia tirucalli)	Male Swiss mice	30 mg/kg (p.o)	PSNL	Antinociception seems likely related with its ability to inhibit the activation and/or release of various inflammatory mediators Systemic, spinal and supraspinal antinociception actions. Ability to interact with cannabinoid system (CB_1 and CB_2 dependent mechanism).
Quintão et al., 2012, Brazil	2 ″- O-rhamnosylswertisin **(21)**/ flavonoid 2(Aleurites moluccana)	Female Swiss and C57/BL6 mice	125, 250 or 500 mg/kg (p.o.)	PSNL	Interacting with opioid system enhancing the descendent-control of pain Interacting dopaminergic and oxidonitrergic system Inflammatory components (neutrophil migration and cytokine release (IL-1 β).
Liu et al., 2012, China	Iridoid glycosides (Paederia scandens)	Adult male SPF SD rats	70, 140, 280 mg/kg (i.g.)	SNI	Antinociception may be partly related to the inhibition of NO/cGMP/PKG signaling pathway
Xu et al., 2012, China	Koumine **(22)** / alkaloid (Gelsemium elegans Benth)	Male ICR mice and male SD rats	0.28, 1.4, 7 mg/kg (s.c.)	CCI SNL	Antinociception may be associated with the up-regulation of allo-pregnanolone in the spinal cord.
Kuwahata et al., 2012, Japan	β-Caryophyllene **(23)**/sesquiterpene (Sigma)	Male mice of ddY strain	4.5, 9.0, 18.0 μg/paw (I.pl.)	PSNL	Activating peripheral CB_2 receptors, but not CB_1 receptors
Amin e Hosseinzadeh, 2012, Iran	Safranal **(24)**/aldehyde (Fluka Chemical)	Adult male Wistar rats	0.025, 0.0 5 and 0.1 mg/kg (i.p.)	CCI	Not registered
	Crocin **(25)**/ Caratenoid (Crocus sativus)		50 mg/kg		Not registered
Kandhare et al., 2012, India	Naringin **(26)**/flavonoid (Sigma)	adult male Wistar rats	20, 40 and 80 mg/kg (p.o.)	STZ - induced diabetic	May exhibit its neuroprotective effect by down regulation of free radical, cytokine including TNF- α Apoptosis and restoration of membrane bound inorganic phosphate activity.

Definition of abbreviations: Route: p.o. = orally, i.p.= intraperitoneal, s.c. = subcutaneous, i.pl.= intraplantar, i.g.= intragastric. Animal:- SD = Sprague-Dawley. Model: STZ = streptozotocin, PSNL = Partial Sciatic Nerve Ligation , CCI = Chronic constriction injury, SNL = spinal nerve ligation, MIA = sodium monoiodoacetate, SNI = spared nerve injury.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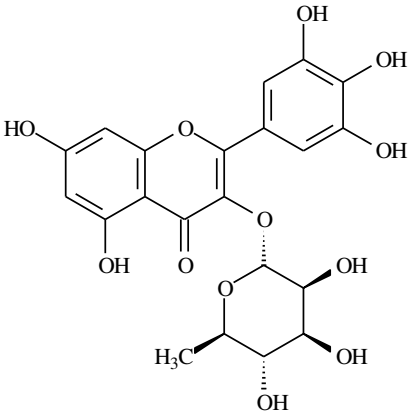	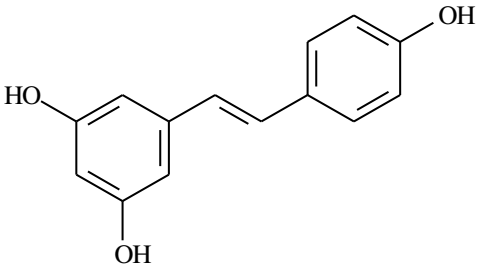
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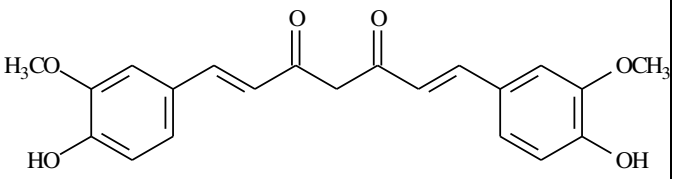	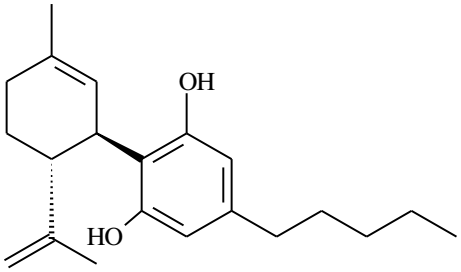
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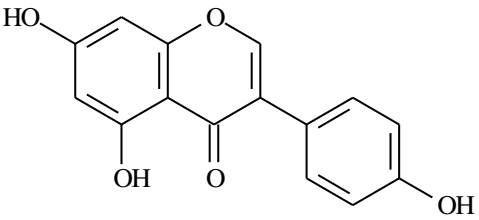	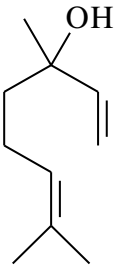
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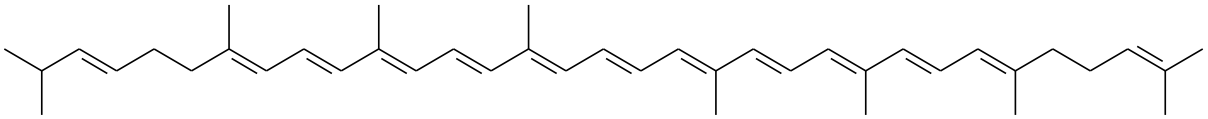	
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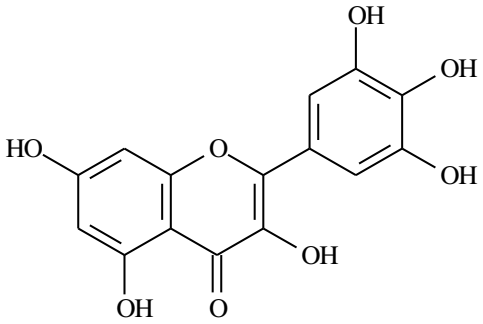	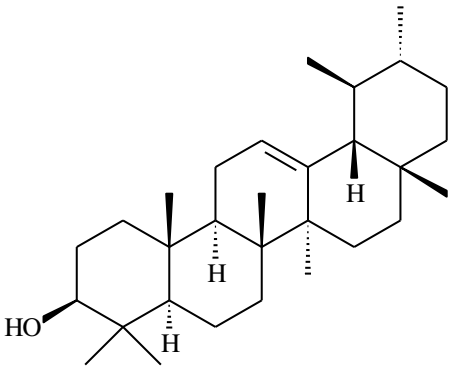
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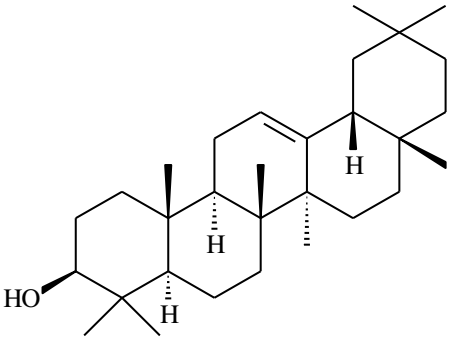	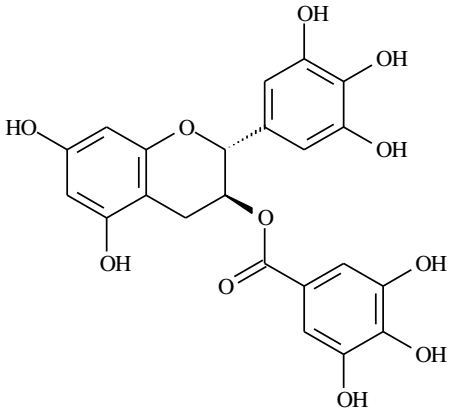
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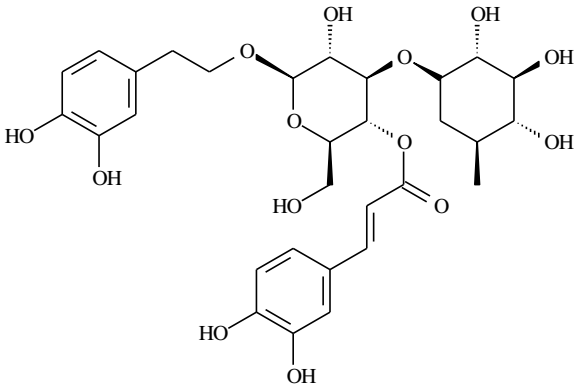	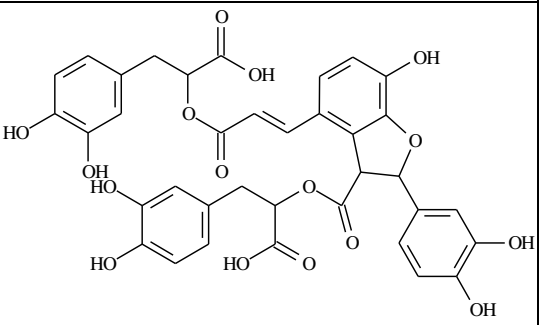
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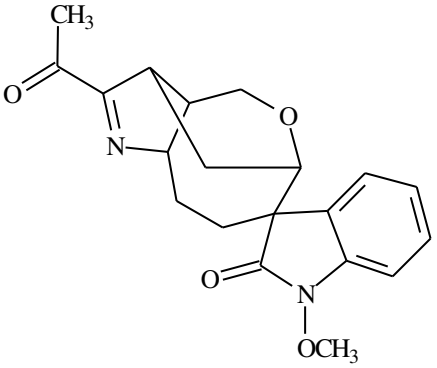	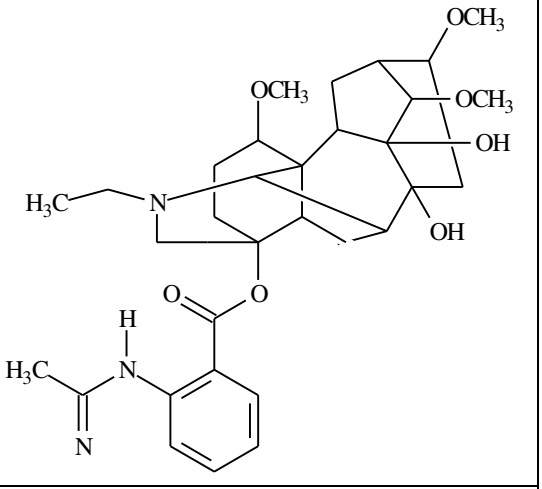
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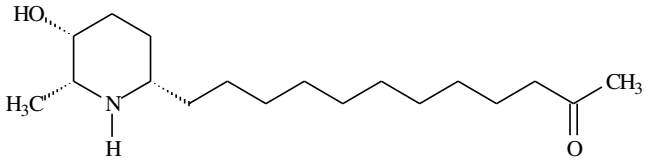	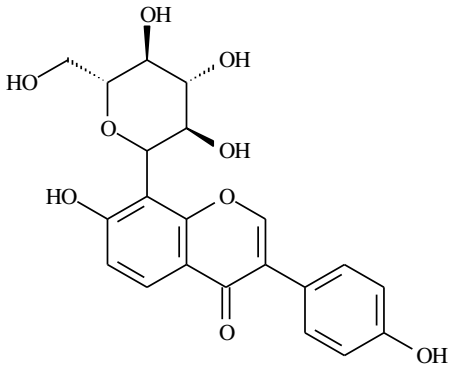
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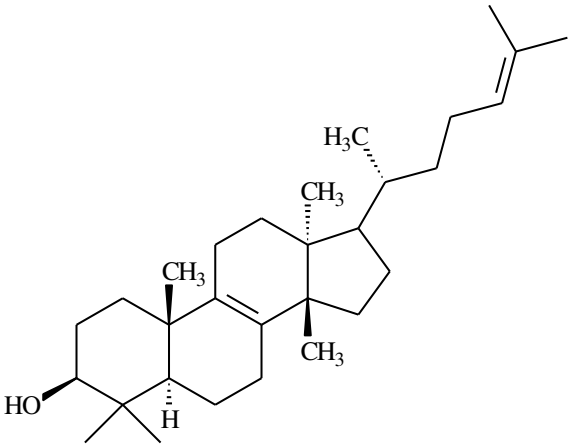	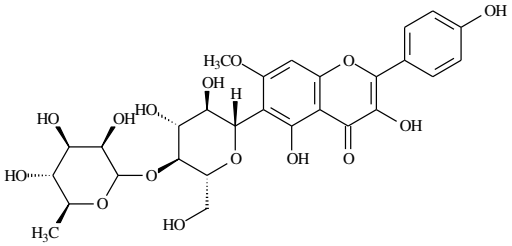
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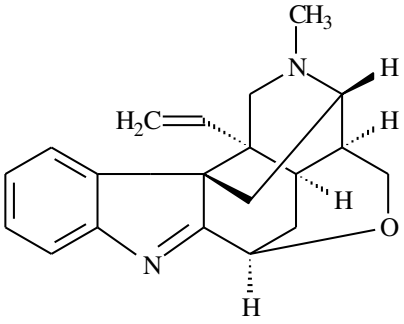	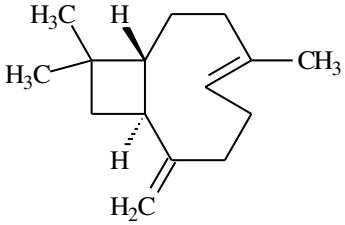
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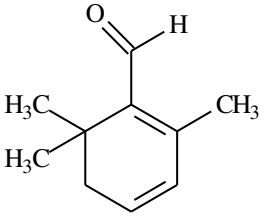	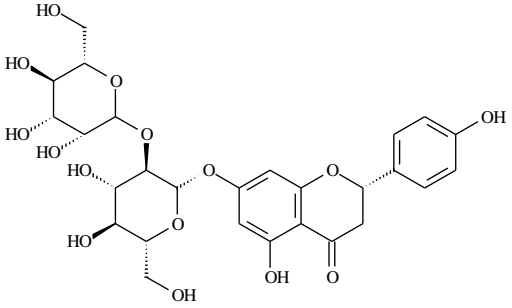
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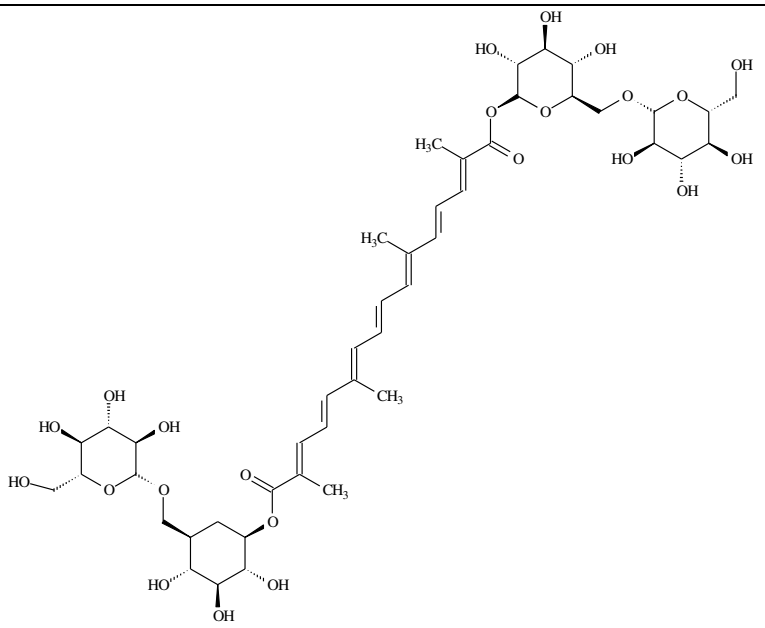

(**25**)

Figure 2. Chemical structures of secondary metabolites

OBJETIVOS

3 OBJETIVOS

3.1 OBJETIVO GERAL

- Avaliar o possível efeito antinociceptivo do acetato de hecogenina em roedores

3.2 OBJETIVOS ESPECÍFICOS

- Avaliar o possível efeito anti-hiperalgésico do acetato de hecogenina na nocicepção inflamatória.

- Avaliar o possível efeito antinociceptivo central do acetato de hecogenina e se este efeito está relacionado com algum déficit motor.

- Investigar o envolvimento de regiões centrais no mecanismo de ação do acetato de hecogenina em camundongos.

- Avaliar o efeito anti-hiperalgésico do acetato de hecogenina, em modelo de dor neuropática.

DESENVOLVIMENTO

4. DESENVOLVIMENTO

4.1 CAPÍTULO 1

Artigo Publicado no **Journal of Natural Products** (Fator de Impacto-JCR 2011: 3,128)

Evidence for the involvement of descending pain-inhibitory mechanisms in the antinociceptive effect of hecogenin acetate

JOURNAL OF NATURAL PRODUCTS

Article

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Evidence for the Involvement of Descending Pain-Inhibitory Mechanisms in the Antinociceptive Effect of Hecogenin Acetate

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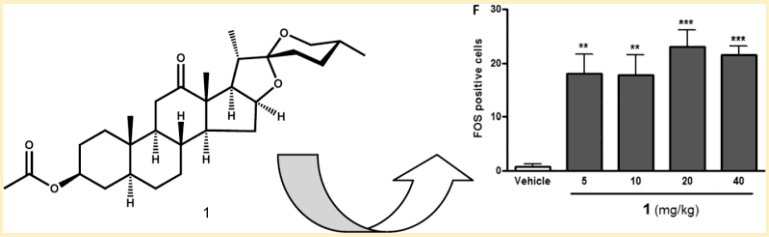

ABSTRACT: Hecogenin is a sapogenin present in the leaves of species from the *Agave* genus, with a wide spectrum of reported pharmacological activities. The present study was undertaken to evaluate whether hecogenin acetate (**1**) has antinociceptive properties and to determine its mechanism of action. The nociceptive threshold was evaluated using the tail flick test in mice. Mice motor performance was evaluated in a Rotarod test. By using Fos expression as a marker of neural activation, the involvement of the periaqueductal gray in **1**-induced antinociception was evaluated. Intraperitoneal administration of **1** (5−40 mg/kg) produced a dose-dependent increase in the tail flick latency time compared to vehicle-treated group ($p < 0.01$). Mice treated with **1** (40 mg/kg) did not show motor performance alterations. The antinociception of **1** (40 mg/kg) was prevented by naloxone (nonselective opioid receptor antagonist; 5 mg/kg), CTOP (μ-opioid receptor antagonist; 1 mg/kg), nor-BNI (κ-opioid receptor antagonist; 0.5 mg/kg), naltrindole (δ-opioid receptor antagonist; 3 mg/kg), or glibenclamide (ATP-sensitive K^+ channel blocker; 2 mg/kg). Systemic administration of **1** (5−40 mg/kg) increased the number of Fos positive cells in the periaqueductal gray. The present study has demonstrated for the first time that **1** produces consistent antinociception mediated by opioid receptors and endogenous analgesic mechanisms.

The sensation of pain plays a critical role as an alerting mechanism and as a protection system against tissue damage. Despite the protective role of pain, the sensation of pain is known to be modified by endogenous pain inhibitory systems, predominantly through the descending noradrenalin, serotonin, and endogenous opioids.[1] The activation of the descending systems markedly reduces the transmission of nociceptive information, and, as a consequence, the severity of pain perception is reduced drastically. Both behavioral and in vivo extracellular recording studies have sought to identify the region that produces such analgesic effects. Several loci are known to produce the antinociceptive effects on pain transmission, including the sensory cortex, thalamus, hypothalamus, midbrain, pons, and rostral ventromedial medulla.[2] Although there are multiple potential target nuclei for modulating pain transmission, following the original report of Reynolds (1969), the periaqueductal gray (PAG) was rapidly established as being important to descending inhibition of spinal nociceptive processing.[3] Through descending projections, the PAG controls spinal dorsal horn pain transmission neurons and mediates both opioid and stimulation-produced analgesia.[4] Advances in knowledge on endogenous analgesic systems have shown attractive pharmacological targets for the development of new analgesic drugs. In fact, drugs widely used clinically for analgesia, such as opioids and antidepressants, act by enhancing the tone of the descending pain inhibition pathways.

Most existing analgesics for persistent pain have a high side effect burden and do not reduce pain in all treated individuals.[5] Therefore, the development of new agents with more powerful analgesic activities and with lesser side effects is, at present, of

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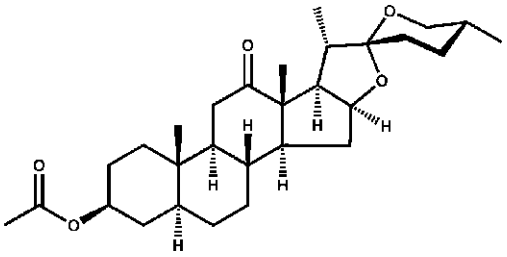
great interest. Since ancient times, natural products have been an important source of analgesic agents.[6] One of the most important analgesic drugs employed in clinical practice today continues to be the alkaloid morphine. Data from 1981 to 2010 indicate that almost forty percent of the drugs approved are based on natural products.[7] In line with these observations, there is currently a strong interest of pharmaceutical companies in plant-derived secondary metabolites for the development of new drugs, with potentially lesser side effects.[7]

Hecogenin is a steroidal sapogenin obtained from plants of the genus Agave (commonly known as "sisal"), belonging to the family Agavaceae, which are distributed widely in tropical and subtropical regions of the world.[8] Agave species possess commercial importance both as a source of industrial fibers and medicinally, as they are used in Chinese folk medicine in the treatment of scabies, tumors, pain, and inflammatory disorders.[9] In addition, hecogenin is an important precursor for the synthesis of steroidal hormones.[10] Moreover, hecogenin has a wide spectrum of pharmacological activities already reported.[11−13] As a function of the antiproliferative activity of hecogenin, the most studied effects of this sapogenin are those related to cancer research.[11] In addition, Cerqueira et al. (2012) showed that hecogenin presents a significant gastroprotective effect that seems to be mediated by K^+_{ATP} channel opening.[12] Peana et al. (1997) demonstrated anti-inflammatory activity of hecogenin by reducing paw edema induced by carrageenin.[13] The antiedematogenic effect of hecogenin was more efficacious than that obtained with indomethacin or dexamethasone at equimolar dose. The present study was undertaken to investigate the antinociceptive properties of hecogenin acetate (1), as well as its effect on the neuronal activity of the periaqueductal gray.



hecogenin acetate (1)

RESULTS AND DISCUSSION

The present study demonstrated, for the first time, the antinociceptive properties of hecogenin acetate (1). The systemic administration of 1, at doses that did not induce any motor performance alteration, inhibited the tail flick reflex, and this effect was prevented by both nonselective and selective antagonists of opioid receptors. In addition, 1 was able to promote neuron activation within the periaqueductal gray, a main site of descending pain-inhibitory pathways. Taken together, these data suggest that 1 induces a long-lasting antinociceptive effect mediated by opioid receptors and activation of endogenous analgesic mechanisms.

The antinociceptive property of 1 was evaluated using the tail flick test, which identifies mainly central analgesics.[18] The intraperitoneal administration of 1 (5−40 mg/kg) produced a dose-dependent enhancement of the reaction time in the tail-flick test (Figure 1; $p < 0.05$), an effect that lasted 5 h. The administration of morphine (5 mg/kg sc), a reference drug

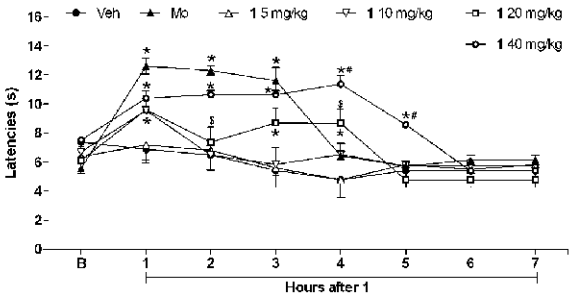


Figure 1. Characterization of the antinociceptive effect of hecogenin acetate (1) in the tail flick test. For the dose−response analysis, the effects of increasing doses of 1 (5 to 40 mg/kg, ip) were tested. To evaluate the time-course of the antinociceptive effect, the thermal nociceptive threshold was evaluated before (B) and up to 7 h following administration of 1 or vehicle (Veh; Tween 20 5%; control group). Morphine (Mo; 5 mg/kg sc), the reference drug, was administered 40 min before the tail flick test. Data are expressed as means ± SEM; n = 6 mice per group. *Significantly different from control group ($p < 0.05$); #significantly different from morphine-treated group ($p < 0.05$); $significantly different from 1 40 mg/kg group ($p < 0.05$). Two-way ANOVA followed by Bonferroni's test was used.

used, caused a significant increase in the latency response for 3 h after administration ($p < 0.05$). In this test, the antinociceptive effect of 1 showed a similar efficacy and long-lasting effect to that of morphine. Moreover, relaxing or motor deficit effects were discarded, since administration of 1 at antinociceptive doses (40 mg/kg) did not affect the motor performance of mice in the Rotarod test (Figure 2). As

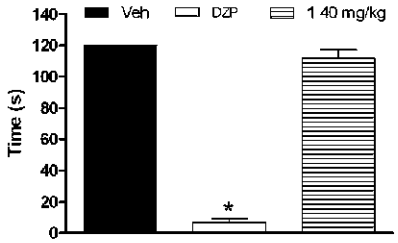


Figure 2. Effects of hecogenin acetate (1) on the Rotarod test. Bar graphs representing the run time on the Rotarod 1 h after the administration of 1 (40 mg/kg) or vehicle (Veh; Tween 20 5%; control group). Diazepam (DZP; 10 mg/kg), the reference drug, was administered 30 min before testing. Data are reported as means ± SEM; n = 6 mice per group. *Significantly different from the control group ($p < 0.001$) as determined by ANOVA followed by Tukey's test.

expected, the central nervous system depressant diazepam (10 mg/kg, ip) reduced the time of mice on the Rotarod after 30 min of treatment with this standard drug ($p < 0.001$). This result indicates that the effects of 1 observed in the nociceptive test do not result from alterations in locomotor activity of the animals, confirming that this sapogenin acetate induces an antinociceptive effect.

Sapogenins, such as hecogenin, are the aglycone nonsugar portions of the saponin molecule used for the semisynthesis of medicinal steroids, such as corticosteroids, sexual hormones,

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and steroid diuretics. As suggested by the chemical structure, **1** exhibits anti-inflammatory properties.[13] Inflammation causes the induction of cyclooxygenase-2, leading to the release of prostanoids, which contribute to the development of peripheral and central sensitization, increasing excitability, and reducing the pain threshold.[19,20] Despite the diverse chemical structures of anti-inflammatory drugs, their antinociceptive effect is mainly due to a common property of prostanoid production inhibition.[21] Accordingly, it is possible that the antinociceptive effect of **1** could also be dependent on its anti-inflammatory properties.[13] On the other hand, in the present study, the antinociceptive effect of **1** was demonstrated in the tail flick test, indicating a central antinociceptive effect for this molecule. The thermal model of the tail flick test is considered to be a spinal reflex, but could also involve higher neural structures.[18,22] These characteristics of this model are helpful tools to investigate the site of action of antinociceptive agents. In addition, from a pharmacological point of view, there is a consensus that this test is efficient only for revealing the activity of opioid analgesics.[18] On the basis of this theory, the effects of the pharmacological blockage of opioid receptors on the antinociceptive activity of **1** were evaluated. The maximal antinociception produced by **1** (40 mg/kg) was completely prevented in mice pretreated with naloxone (5 mg/kg ip; 15 min before), a nonselective opioid receptor antagonist, suggesting an opioid-like activity for this sapogenin acetate (Figure 3a). Similarly, the administration of the μ-opioid receptor antagonist CTOP (1 mg/kg ip) 30 min after the administration of **1** blocked the observed antinociceptive effect (Figure 3b). Pretreatment with the κ-opioid receptor antagonist nor-BNI (0.5 mg/kg sc; 15 min before) or the δ-opioid receptor antagonist naltrindole (3 mg/kg sc; 5 min before) also prevented **1**-induced antinociception (Figure 3b). In addition, the administration of the opioid receptor antagonists, at a similar range of dose, did not modify per se the reaction time in the tail-flick test (data not shown). These results suggest that opioid receptors play a major role in the antinociceptive effect of **1**.

It was recently demonstrated that **1** shows gastroprotective effects dependent on ATP-sensitive K^+ (K^+_{ATP}) channels, since its effects were reversed by pretreatment with glibenclamide, a potent blocker of these channels.[11] Several in vivo studies have suggested that K^+_{ATP} channels are involved in opioid-induced analgesia. Glibenclamide, dose-dependently decreased the antinociceptive response of morphine, levorphanol, methadone, and buprenorphine.[23−25] On the other hand, K^+_{ATP} channel openers are able to potentiate the antinociceptive response of morphine.[26−28] In addition, previous findings implicate the release of endogenous opioids in the mediation of antinociceptive effects of K^+_{ATP} channel openers.[26] Aiming to investigate this idea, the effects of pharmacological blockage of K^+_{ATP} channels on the antinociceptive activity of **1** were evaluated. The antinociceptive effect of **1** (40 mg/kg) was completely prevented in mice pretreated with glibenclamide (2 mg/kg ip; 30 min before) (Figure 4). The function of **1** in K^+_{ATP} channels reinforces the hypothesis of the involvement of the opioid system in their mechanisms of antinociception proposed in the present work.

Anatomical and physiological studies conducted in the 1960s and 1970s identified the periaqueductal gray and its descending projections to the spinal cord dorsal horn as a primary anatomical pathway that mediates descending pain-inhibitory mechanisms.[3,29] The PAG projects to the ventromedial

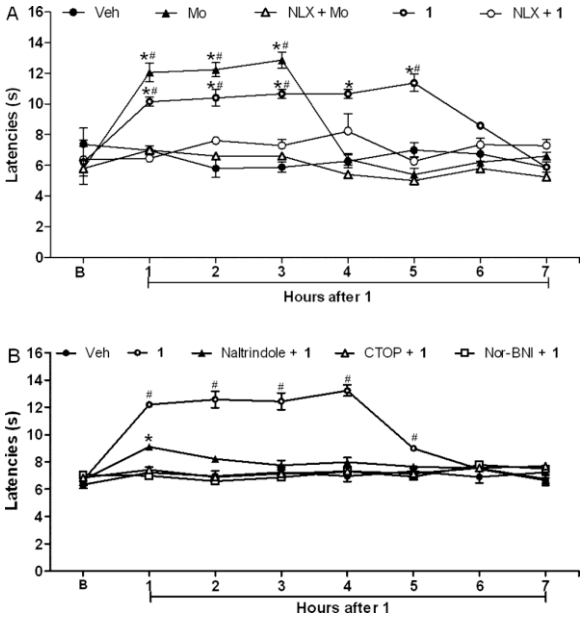

Figure 3. Effects of the pharmacological blockage of opioid receptors on the antinociceptive effect of hecogenin acetate (**1**). The thermal nociceptive threshold was evaluated before (B) and up to 7 h following administration of **1** (40 mg/kg) or vehicle (Veh; Tween 20 5%; control group). (A) Effects of naloxone, a nonselective opioid receptor antagonist, on the antinociceptive effect of **1**. Naloxone (NLX; 5 mg/kg ip) was administered 15 min before the administration of **1**, morphine, or vehicle. Morphine (5 mg/kg sc) was administered 40 min before the tail flick test. (B) Effects of the μ-opioid receptor antagonist CTOP (1 mg/kg ip; 30 min after **1**), the κ-opioid receptor antagonist nor-BNI (0.5 mg/kg sc; 15 min before **1**), and the δ-opioid receptor antagonist naltrindole (3.0 mg/kg sc; 5 min before **1**) on the antinociceptive effect of **1**. Data are expressed as means ± SEM; $n = 6$ mice per group. Two-way ANOVA followed by Bonferroni's test was used. Panel A: *Significantly different from control group ($p < 0.05$); #significantly different from naloxone-treated groups ($p < 0.05$). Panel B: *Significantly different from control group ($p < 0.05$); #significantly different from remaining groups ($p < 0.05$).

medulla, which, in turn, sends its output to dorsal horn laminae important in nociceptive functions.[30] Since the PAG has a pivotal role in endogenous analgesia mechanisms and is also recognized as the central site of action of analgesic opioids, in the present study the ability of **1** to promote activation of neurons within the PAG was evaluated.[29,30] For this purpose, immunohistochemical detection of the protein product (Fos) of the *c-fos* proto-oncogene was carried out. Vehicle-treated mice showed low levels of Fos expression in the periaqueductal gray (Figure 5A and F). However, following systemic administration of **1** (5−40 mg/kg), there was a major increase in the number of Fos-positive cells in the PAG when compared to the control group ($p < 0.001$; Figure 5B, C, D, and E). Therefore, by using Fos expression as a neural marker, it was possible to suggest that **1** induces PAG activation. The immediate early gene *c-fos* is rapidly and transiently expressed in neurons in response to stimulation.[31] The gene encodes for the nuclear protein Fos, and the levels of the protein peak about 2 h after induction of gene transcription.[32] In line with literature data, the effect of **1** on Fos expression was observed 2 h after the drug injection. The involvement of PAG in the

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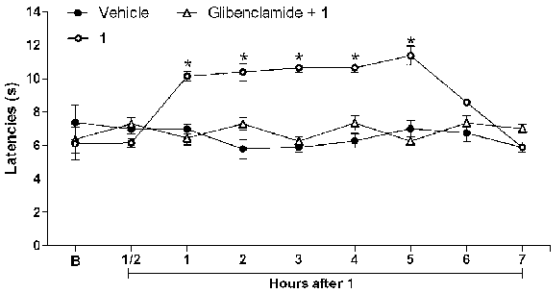

Figure 4. Effects of the pharmacological blockage of ATP-sensitive K^+ (K^+_{ATP}) channel on the antinociceptive effect of hecogenin acetate (1). The thermal nociceptive threshold was evaluated before (B) and up to 7 h following administration of 1 (40 mg/kg) or vehicle (Veh; Tween 20 5%; control group). Figure shows the effects of glibenclamide, a K^+_{ATP} channel blocker, on the antinociceptive effect of 1. Glibenclamide (2 mg/kg ip) was administered 30 min before the administration of 1. Data are expressed as means ± SEM; n = 6 mice per group. Two-way ANOVA followed by Bonferroni's test was used. *Significantly different from remaining groups ($p < 0.05$).

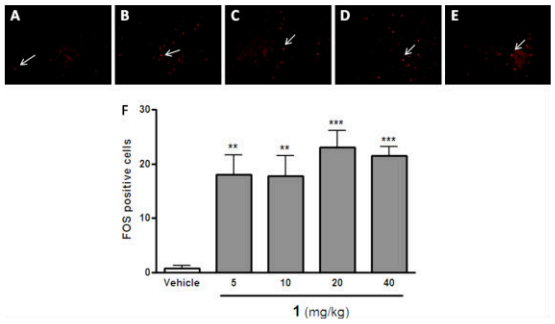

Figure 5. Imunofluorescence for Fos protein. The white arrows point to Fos-positive neurons in the periaqueductal gray. Vehicle (A) or hecogenin acetate (1) (B: 5, C: 10, D: 20, E: 40 mg/kg) was administered by the intraperitoneal route 2 h before perfusion. The bar graph shows (below and right side) average FOS-positive cells compared with the vehicle-treated group (white bar). Data are reported as means ± SEM; n = 6 mice per group, **$p < 0.01$ and ***$p < 0.001$ vs control group as determined by ANOVA followed by Tukey's test.

antinociceptive effect of hecogenin was not presently evaluated, but data from the literature have established the measure of c-fos expression as a valid tool for the study of the neural correlates of nociception.[33] Visualization of c-fos expression allows a precise anatomical record of neuronal populations that are activated during nociceptive processing and has advanced the understanding of where many analgesic drugs and endogenous analgesic systems act to reduce pain.[34]

In conclusion, the present study has demonstrated for the first time that systemic administration of 1, at doses that did not induce motor performance alterations, produced a consistent antinociceptive effect, possibly mediated by the opioid system and descending pain-inhibitory pathways activation. However, a more in-depth evaluation of the mechanisms involved in 1-induced antinociception is still necessary.

EXPERIMENTAL SECTION

General Experimental Procedures. Experiments were performed on male Swiss Webster mice (20−25 g) obtained from the Animal Facilities of Centro de Pesquisas Gonçalo Moniz. Animals were housed in temperature-controlled rooms (22−25 °C), under a 12:12 h light−dark cycle, with access to water and food ad libitum until use. All behavioral tests were performed between 8:00 A.M. and 5:00 P.M., and animals were used only once. Animal care and handling procedures were in accordance with the International Association for the Study of Pain guidelines for the use of animals in pain research and the Institutional Animal Care and Use Committee FIOCRUZ CPqGM 009/2011.[14] Every effort was made to minimize the number of animals used and to avoid any discomfort.

Test Compounds and Administration. The test compounds used in this study were hecogenin acetate (1), glibenclamide (ATP-sensitive K^+ channel blocker), naloxone (nonselective antagonist of opioid receptors), naltrindole (δ-opioid receptor antagonist), and nor-binaltorphimine (Nor-BNI; κ-opioid receptor antagonist), which were obtained from Sigma Chemical Company (St. Louis, MO, USA). D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide (CTOP; μ-opioid receptor antagonist) was purchased from Tocris Bioscience (Bristol, UK), and diazepam and morphine were from Cristália (Itapira, São Paulo, Brazil). Hecogenin acetate (95% purity) was dissolved in Tween 20 5% plus physiological saline, and the remaining compounds were dissolved in physiological saline. The test compounds were administered by intraperitoneal (ip) or subcutaneous (sc) routes at doses and conditions previously standardized.[15]

Tail Flick Test. The warm-water tail withdrawal test in mice was conducted as described previously, with minor modifications.[16] Before the day of the experiment, each animal was habituated to the restraint cylinder for five consecutive days (20 min per day). On the day of the experiment, mice were placed in a restraining cylinder, and the tail tip (2 cm) was immersed in a water bath at 50 ± 0.5 °C. The latency for the tail withdrawal reflex was measured. Each trial was terminated after 16 s to minimize the probability of skin damage. The tail flick latency was measured before (baseline) and after treatment.

Assay of Motor Function. To evaluate possible nonspecific muscle-relaxant or sedative effects of hecogenin acetate (1), mice were submitted to the Rotarod test.[17] The Rotarod apparatus (Insight, Ribeirão Preto, Brazil) consisted of a bar with a diameter of 3 cm, subdivided into five compartments. The bar rotated at a constant speed of 6 rpm. The animals were selected 24 h previously by eliminating those mice that did not remain on the bar for two consecutive periods of 120 s. Animals were treated with diazepam (10 mg/kg, ip), 1 (40 mg/kg, ip), or vehicle (200 μL, ip) and 40 min afterward were placed on the rotating rod. The resistance to falling was measured up to 120 s. The results are expressed as the average time (s) the animals remained on the Rotarod in each group.

Immunohistochemical Studies. Two hours after the intraperitoneal injection of 1 (5, 10, 20, or 40 mg/kg) or vehicle, mice (n = 6, per group) were perfused, and the brains were collected for immunofluorescence processing for Fos protein. Frozen serial transverse sections of 20 μm containing the periaqueductal gray were collected on gelatinized glass slides. The tissue sections were stored at −70 °C until use. The sections were washed with phosphate buffer (0.01 M) saline isotonic (PBS) three times for 5 min and incubated with 0.1 M glycine in PBS for 10 min. Nonspecific protein binding was blocked by incubation of the sections for 30 min in a solution containing 1% bovine serum albumin. After this, sections were incubated overnight with rabbit anti-Fos (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as primary antibodies. Afterward, the sections were incubated for 1 h with donkey anti-rabbit Alexa Fluor 594 as secondary antibodies (Molecular Probes, Eugene, OR, USA). The coverslip was mounted with Fluoromount G (Electron Microscopy Sciences, Hatfield, PA, USA). As an immunohistochemistry control for nonspecific labeling, sections were incubated without primary antibody. After each stage, slides were washed with PBS three times for 5 min.

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Acquisition and Analyses of Images. Pictures from the periaqueductal gray were acquired for each animal (n = 6, per group) with an Axioskop 2 Plus (Carl Zeiss, Germany). Neurons were counted by the free software Image J (National Institute of Health) using a plugin (written by the authors) that uses the same level of label intensity to select and count the Fos-positive cells.

Statistical Analysis. All data are presented as means ± standard error of the mean (SEM) of measurements made on six animals in each group. All data were analyzed using the Prism 5 computer software (GraphPad, San Diego, CA, USA). Comparisons across three or more treatments were made using one-way ANOVA with Tukey's post hoc test or repeated measure two-way ANOVA with Bonferroni's post hoc test used when appropriate. Statistical differences were considered to be significant at $p < 0.05$.

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Notes

The authors declare no competing financial interest.

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4.2 CAPÍTULO 2

Artigo submetido ao Journal of Pharmacy and Pharmacology (Fator de Impacto-JCR 2011: 2,175)

Antinociceptive activity of hecogenin acetate, a steroidal sapogenin-acetylated, in experimental inflammatory pain: a possible involvement of the spinal cord inhibition

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Abstract:

Hecogenin is a steroidal sapogenin largely drawn from the plants of the genus Agave, commonly known as 'sisal', and is one of the important precursors used by the pharmaceutical industry for the synthesis of steroid hormones. Our group has recently reported that hecogenin acetate (HA), a steroidal sapogenin-acetylated, produced antinociception in the tail flick test, as well as its effect with the involvement of descending pain-inhibitory mechanisms. Now, we evaluate the antihyperalgesic activity of HA in mice in inflammatory models. Acute pretreatment with HA (5, 10, or 20 mg/kg; i.p.) inhibited the development of mechanical hyperalgesia induced by carrageenan, TNF-α, dopamine and PGE_2. Additionally, the immunofluorescence data demonstrated that acute pretreatment with HA, at all doses tested, significantly inhibited Fos-like expression in the spinal cord dorsal horn normally observed after carrageenan-inflammation. Moreover, HA did not affect the motor performance of the mice as tested in the Rota rod test. The present results suggest that HA attenuates mechanical hyperalgesia, at least in part, by blocking the neural transmission of pain at the spinal cord levels.

Key words: Hecogenin acetate, mechanical hyperalgesia, pain, spinal cord, steroidal sapogenin.

Introduction

Pain management remains one of the greatest challenges of contemporary medicine, nevertheless, there are a relatively limited list of treatment options for pain [1]. Although a considerable number of analgesic drugs are available for the treatment of painful disorders, the search for development of new compounds as therapeutic alternatives continues since the available analgesic drugs exert a wide range of side effects [2,3]. In this regard, opioids and nonsteroidal anti-inflammatory drugs (NSAIDs) have been the main stay of pain treatment. Presently, there is concern that success in the development of new analgesic agents is limited [1].

Although the expansion of synthetic medicinal chemistry in the last decades caused the proportion of new drugs based on natural products to drop to ~50%, 13 natural product–derived drugs were approved in the U.S. Food and Drug Administration (FDA) between 2005 and 2007, with five of them being the first members of new classes [4]. For the treatment of pain the latest example of the natural product approved by FDA is Ziconotide (Prialt®), a peptide toxin obtained from Conus magus (marine gastropod mollusk in the family Conidae), and it is the first member in the new drug class of selective N-type voltage-sensitive calcium-channel blockers [5]. Currently, because of their relatively low cost and easy availability in several countries (mainly developing countries), natural products could be used as synthetic models of more selective and powerful drugs [6,7].

Hecogenin is a steroidal sapogenin largely drawn from the plants of the genus Agave (commonly known as ‘sisal’), and belongs to family Agavaceae, widely distributed in tropical and subtropical regions throughout the world [8]. ‘Sisal’ species possess both commercial importance (as source of industrial fibers) and medicinal importance and they have been used in Chinese medicine for the treatment of scabies

and tumors, as well as for painful and inflammatory conditions [9,10]. The steroidal sapogenins obtained from 'sisal' species (mainly diosgenin and hecogenin) are important precursors used by the pharmaceutical industry for the synthesis of steroid hormones, such as adrenal cortical hormones (cortisone, cortisol, prednisolone, prednisone, dexamethasone, betamethasone, triamcinolone, etc.), sexual hormones (progesterone), and protein anabolic hormones (stanozolol, methandienone) [11,12,13]. However, there is little scientific information about the biological properties of hecogenin in painful and inflammatory conditions. Peana et al. [14] demonstrated hecogenin reduced paw edema induced by carrageenin, but also produced gastric mucous lesions in higher doses. Cerqueira et al. [15] proposed that the anti-inflammatory property of hecogenin was produced by COX-2 inhibition. Conversely, in Cerqueira et al. [15] study pretreatment with hecogenin produced significant gastroprotective effects mediated by K^+_{ATP} channels.

Recently, our group reported that hecogenin acetate (HA), a steroidal sapogenin-acetylated, possesses antinociceptive activity using the tail-flick test [43]. Now, the antinociceptive effect of HA was investigated in inflammatory hyperalgesia models on mice and examined HA-evoked cFos immunoreactivity in spinal cords levels. We hypothesized that HA may be acting by spinal cord inhibitory mechanisms to produces anti-hyperalgesic profile.

Materials and Methods

Animals

All experimental protocols were approved by the Animal Care and Use Committee (CEPA/UFS # 04/12) at the Federal University of Sergipe, and handling

procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH - National Institutes of Health) for the use of animals in pain research [16]. Male Swiss mice (32-39 g), 2-3 months of age, were used throughout this study. The animals were randomly housed in appropriate cages at 21 ± 2 ºC on a 12 h light/dark cycle (lights on from 6:00 a.m. to 6:00 p.m.) with free access to food (Purina®, Brazil) and water. Before the experiments, the animals were acclimatized to the laboratory for at least 1h. Mice were used only once in each test. Experiments were carried out between 9:00 a.m. and 2:00 p.m. in a quiet room. All experiments involving the behavioral analysis were carried out by the same visual observer and in a double-blind manner.

Drugs and Reagents

λ-Carrageenan, tumor necrosis factor-alpha (TNF-α), prostaglandin E_2 (PGE_2), dopamine (DA), ethylenediamine tetraacetic acid (EDTA), Tween 80, fluoromount G, glycine and bovine serum albumin (BSA) and hecogenin acetate (HA, ~90% purity) (Fig. 1) were purchased from Sigma (Sigma, St. Louis, MO, USA). Indomethacin and dipyrone were purchased from União Quimica (Brazil). c-Fos Antibody, a rabbit polyclonal IgG, was obtained from Santa Cruz Biotechnology (USA) and Alexa Fluor® 488, a Donkey Anti-Rabbit IgG (H+L), was obtained from Life technology (USA).

Hyperalgesic stimulus and nociceptive threshold evaluation

Mechanical hyperalgesia was tested in mice as reported by Cunha et al. [17], with alterations as previously published [18]. In a quiet room, mice were placed in acrylic cages (12 x 10 x 17 cm) with wire-grid floors 15-30 min before starting the test. This method consisted of evoking a hindpaw flexion reflex with a hand-held force

transducer (electronic anesthesiometer, Model EFF 302, Insight®, Brazil) adapted with a polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hindpaw with a gradual increase in pressure. The end point was characterized by the paw withdrawal followed by clear flinching movements. After this response, the intensity of the pressure was automatically recorded. The intensity of stimulus was obtained by averaging four measurements performed with minimal intervals of 3 min. The animals were tested before and after treatments.

Mice were divided into five groups (n = 8, per group), which were treated with vehicle (saline + tween 80 0.2%; i.p.), hecogenin acetate (5, 10 or 20 mg/kg; i.p.), indomethacin (10 mg/kg; i.p.) or dipyrone (60 mg/kg; i.p.). Thirty min after treatment, 20 μL of carrageenan (300 μg/paw), PGE_2 (100 ng/paw), DA (30 μg/paw) or TNF-α (100 pg/paw) were injected subcutaneously into the subplantar region of the hindpaw, as described by Cunha et al. [17] and Villarreal et al. [19]. The degree of hyperalgesia was evaluated at 0.5, 1, 2, and 3 h after the injection of hyperalgesic agents.

Motor function assay (rota-rod test)

To evaluate the possible non-specific muscle-relaxant or sedative effects of hecogenin acetate in the doses used, mice were submitted to the rota-rod test (AVS®, Brazil) according to Dunham and Miya [21]. The animals were selected 24 h previously by eliminating those mice which did not remain on the bar for two consecutive periods of 240 s. Mice were pre-treated with diazepam (DZP, 3 mg/kg, i.p., reference drug), hecogenin acetate (5, 10 or 20 mg/kg, i.p.) or vehicle (saline + Tween 80 0.2%, i.p.) and 1 h later were placed on a rotating rod. The latency to falling was measured up to 180 s. The results are expressed as the average time (s) during which the animals remained on the rota-rod apparatus in each group.

Immunofluorescence

Ninety minutes after the intraperitoneal injection of hecogenin acetate at doses of 5, 10 and 20 mg/Kg or vehicle (Saline + Tween 80 0.2%), the animals (n = 6, per group) were perfused with phosphate buffer (0.01 M) saline isotonic (PBS) followed by 10% buffered formalin (100 mM). The spinal cord (L4-L6) were removed and stored at -80°C for the imunofluorescence against Fos protein.

The protocol for immunofluorescence was based on prior [22] [23]. Frozen serial transverse sections (20 µm) of the whole spinal cord were collected on gelatinized glass slides. After washing with PBS, the slices were incubated with 0.1 M glycine in PBS for 10 min. Non-specific protein binding was blocked by the incubation of the sections for 30 min in a solution containing 2% BSA. Then, the sections were incubated overnight with rabbit anti-Fos as primary antibodies (1:2000). Afterwards, the sections were incubated for 2 hr with donkey anti-rabbit IgG -Alexa Fluor 594 (1:2000). Sections were cover slipped with Fluoromount G. As a control for non-specific labeling, sections were incubated without primary antibody. After each stage, slides were washed with PBS five times for 5 min.

Acquisition and analyses of images

Light level photomicrographs of spinal cord sections were acquired for each animal with an Axioskop 2 plus, Carl Zeiss®, Germany. Neurons were counted by the free software Image J (National Institute of Health) using a plug-in (written by the authors) that uses the same level of label intensity to select and count the Fos-positive cells.

INSERT FIGURE 1

Statistical analysis

Data are presented as means ± standard error of the mean (SEM) of measurements made on 6-8 animals in each group. Comparisons between three or more treatments were made using one-way analysis of variance (ANOVA) followed by Tukey's test. In all cases, differences were considered significant if $p < 0.05$. All statistical analyses were carried out using Graph Pad Prism 5.0 (Graph Pad Prism Software Inc., San Diego, CA, USA).

Results

Effect of hecogenin acetate on Carrageenan -induced mouse paw mechanical hyperalgesia

As showed in Fig. 2, intraplantar injection of carrageenan (300 µg/paw) induced a significant increase in the number of responses to 0.6g force applied to the inflamed hindpaw when compared to baseline. The increased number of responses were maintained from 30 to 180 min after the carrageenan administration. The systemic administration of hecogenin acetate (5, 10 or 20 mg/kg, i.p.) produced an anti-hyperalgesic effect in this model. Specifically mice treated with hecogenin acetate, all doses, 30 min before carrageenan administration exhibited a significant reduction in the number of responses to repeated application of the 0.6 g mechanical force at all evaluated times, when compared with animals vehicle-treated (control group). As expected, the reference drug (indomethacin, 10 mg/kg, i.p.) showed reductions in the number of responses to the 0.6 g force. (Fig. 2A). The animals group that received saline in the sub plantar region, instead of carrageenan, did not present any alteration on the threshold of sensitivity towards the mechanical stimuli (data not shown).

INSERT FIGURE 2

Effect of hecogenin acetate on TNF-α, dopamine or PGE_2-induced mouse paw mechanical hyperalgesia

The inhibitory effect of hecogenin acetate (5, 10 or 20 mg/kg, i.p.) on the mechanical hyperalgesia induced by TNF-α is shown in Fig. 2B. Hecogenin acetate (5, 10 or 20 mg/kg) was able to reduce mechanical hyperalgesia induced by TNF-α, when compared with animals of the control group (vehicle-treated), similarly to the reference drug (indomethacin, 10 mg/kg, i.p.). Fig. 2C shows the inhibitory effect of hecogenin acetate on the mechanical hyperalgesia induced by dopamine. Hecogenin acetate, in a higher dose, was able to reduce mechanical hyperalgesia induced by dopamine, when compared with animals of the vehicle group. Intraplantar administration of PGE_2 induced a marked mechanical hypersensitivity that was significantly reduced by dipyrone (60 mg/kg, i.p.) and by hecogenin acetate at 5-20 mg/kg, as shown in Figure 2D.

Evaluation of the motor activity

In the rota-rod test, the pre-treatment with hecogenin acetate, all doses, did not show any significant motor performance change when compared to control animals (Fig. 3). As expected, the reference drug (diazepam, 3 mg/kg, i.p.) significantly reduced the time during which animals remained on the rota-rod apparatus.

INSERT FIGURE 3

Effect of hecogenin acetate on Fos analysis in spinal cord areas

Ninety minutes after the intraperitoneal injection of hecogenin acetate, the average number of neurons showing Fos protein in the spinal cord dorsal horn was significantly ($p < 0.05$) reduced (Fig. 4) at doses of 5, 10 and 20 mg/Kg when compared with control (Vehicle).

INSERT FIGURE 4

Discussion

The current study shows that systemic administration of hecogenin acetate reduced inflammatory hyperalgesia and its reduce fos expression in the dorsal horn of the spinal cord normally produced by carrageenan suggesting it reduces central excitability.

Hyperalgesia induced by i.pl. injection of carrageenan is widely used for evaluating new anti-hyperalgesic drugs in rodents [19]. In experiments using mice, injection of carrageenan into the plantar surface of animal hind paws produced inflammation and hyperalgesia with similar temporal profile [24]. In this model, there is the occurrence of non-immunereaction, involving inflammatory mediators, including arachidonic acid products (PGE_2), mast cells products (histamine, 5-HT), neuropeptides, cytokines (IL-1β and TNFα), NO, nerve growth factor (NGF), leukotrienes B4 (LTB4) and transcription factors (NF-κB) [25,26]. According to Cunha et al. [27], this cascade of signalization leads to the release of prostanoids and sympathomimetic amines. Therefore, those final inflammatory mediators can activate peripheral Aδ and C fiber sensory nerve terminals, thus causing the release of substance P and neurokinin A, leading to increases in local blood flow and vascular permeability [28].

Cytokines produced after i.pl. injection of carrageenan may exert in direct cytotoxic effects by the release of NO, reactive oxygen species, eicosanoids and excitatory amino acids (EAA - glutamate and aspartate) EAA [29]. Alternatively, IL-1β induced by carrageenan might sensitize spinal neurons through the induction of nociceptive neuropeptides expression, such as substance P (SP) and nerve growth factor (NGF). It also has been shown that nociceptive stimulus induced by glutamate, PGs, histamine or 5-HT carrageenan-induced release resulted on direct nociceptors sensitization, culminating with simultaneous thermal and mechanical hyperalgesia [30]. We showed that acute hecogenin acetate administration, all doses, was able to reverse the hyperalgesic response induced by carrageenan, thus suggesting a possible inhibitory effect on the inflammatory cascade.

It is well know that cytokine TNF-α is recognized as a potent pro-inflammatory endogenous substance, which is rapidly produced in large quantities by macrophages in response to inflammatory stimuli such as bacterial infection [31]. TNF-α interacts with target cells through high-affinity membrane receptors, as tumor necrosis factor receptor type 1 (TNFR1 or p55) and type 2 (TNFR2 or p75) [32]. Thus, TNF-α-induced hyperalgesia is mediated by prostanoids and sympathetic amines. We demonstrated that hecogenin acetate was able to reverse the hyperalgesic response induced by TNF-α, thus suggesting a possible inhibitory effect on the inflammatory cascade, as observed with the treatment with indomethacin.

Taking those findings into account, we evaluated whether the antinociceptive activity of hecogenin acetate involves the blockade of sensitization or activation of the nociceptor through evaluation of its effect on hyperalgesia induced by PGE_2 and dopamine [19]. As the hyperalgesia induced by these mediators is independent of the final production of other inflammatory mediators or recruitment of cells, such as

neutrophils [33] and as it has been demonstrated that hecogenin acetate administration was able to maintain the baseline nociceptive threshold, we can then suggest the possibility that hecogenin acetate interacts with dopamine or EP receptors; consequently, the paths of its analgesic effects may be through the action not only at the inflammatory level but also from a possible involvement of neuronal pathways [34], as suggested by Brito et al. [34] and Gama et al. [43].

In fact, the acute pretreatment with hecogenin acetate increased threshold sensitivity towards mechanical stimuli in carrageenan-, TNF-α, PGE_2- or dopamine-induced mechanical hyperalgesia, this compound is suggested to have produced an inhibition of the inflammatory cascade. Previously, Peana et al. [14] and Cerqueira et al. [15] have shown the antiedematogenic and anti-inflammatory effects of hecogenin, and they demonstrated that anti-inflammatory activity of hecogenin may be involvement inhibitory of cytokines pathway. Additionaly, Cerqueira et al. [15] proposed that hecogenin increased COX-2 expression in ethanol-induced gastric ulcer in rodents and who also suggest that hecogenin produces beneficial effect in gastric injury through mechanisms involving the inhibition of inflammatory cell infiltration and lipid peroxidation, up-regulation of the COX-2/PG pathway and K^+_{ATP} channels.

As it is well known, peripheral or central opioid receptor activation could lead to the decrease of the pain sensation towards an inflammatory stimulus, through the NO-cGMP-K^+_{ATP} channel pathway activation [35]. Based on both suggestions that hecogenin acetate promotes regulation of K^+_{ATP} channels and the inhibitory effect of opioids on the spinal nociceptive transmission, and as our group has recently demonstrated, for the first time, that systemic administration of hecogenin acetate, at doses that did not induce motor performance alterations, produced consistent antinociceptive effect, probably mediated by opioid system and descending pain-

inhibitory pathways activation [43], we investigated whether analgesic-like profile of hecogenin acetate was due to the involvement of spinal cord dorsal horn pathways through immunohistochemical approach.

The superficial dorsal horn of the spinal cord, particularly substantia gelatinosa (SG), is a major projection site of small-diameter afferent nerve fibers that predominantly transmit nociceptive signals. SG neurons also receive descending inputs from the brainstem [36]. Baba et al. [37] demonstrated, using an in vitro spinal cord slice preparation, that peripheral inflammation can facilitate A-beta fiber-mediated synaptic inputs to dorsal horn of the spinal cord, mainly SG, and also produce an increase in c-fos expression.

The protooncogene c-fos, when activated, makes the immunologically detectable nuclear protein Fos [38]. A striking attribute of Fos is that it is rapidly expressed in central neurons after noxious stimuli [39]. Previous studies demonstrated that the increased c-fos expression is a transient reaction of spinal neurons in painful conditions, as in chronic pain [39, 40]. The causes of that spinal cord nociceptive neuronal hyperactivity during certain types of pain remain unclear. It may derive from the constant barrage of peripheral input [41], but may also reflect impairments of descending modulation, as previously showed by hecogenin acetate [43]. Anyway, the value of the analysis of Fos expression to monitor the nociceptive activity of large neuronal populations is solid [39].

In addition, CNS-depressant drugs, such as gabapentin, frequently used as analgesic in some types of painful conditions, inhibit glutamatergic excitatory neurotransmission at the spinal dorsal horn and decrease Fos expression when they produce analgesic effect [42]. Thus, our results suggests that the inhibition of neuronal hyperactivity at the spinal cord dorsal horn (by a significantly decrease of Fos

expression) could account for the analgesic efficacy of hecogenin acetate, in all doses tested, in carrageenan-induced inflammatory nociception. Hence, it may also decrease neuronal activation at the spinal cord horn, by depressing descending facilitation through periaqueductal gray (PAG) activation, as previously demonstrated by our group [43].

Earlier studies suggested that the CNS depression and the non-specific muscle relaxation effects can reduce the response of motor coordination and might invalidate the behavioral test results, including mechanical hyperalgesic tests [18,33]. Since Brito et al. [34] showed the anxiolytic and antidepressant effects of hecogenin and as these activities can induce impair in motor coordination, we showed that acute treatment with hecogenin acetate, at the doses tested, did not have any performance alteration in the rota-rod apparatus.

Together, our results clearly indicate that hecogenin acetate displays significant anti-hyperalgesic effect in animal protocols. The precise mechanism of action by which hecogenin acetate promotes its effects is not clear, but the compound's ability to modulate spinal cord dorsal horn pathways is likely one of the possible mechanisms. However, it is possible that another central or peripheral mechanism, not studied in present work, may be related to the analgesic effect of hecogenin acetate. Therefore, this steroidal sapogenin may be of potential interest in the development of new clinically relevant drugs for the management of painful conditions.

Acknowledgments

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Declaration of interest: The authors report no conflicts of interest.

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FIGURES

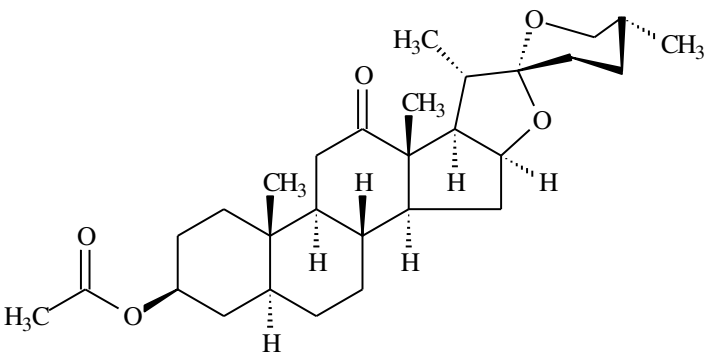

Figure 1 – Chemical structure of hecogenin acetate

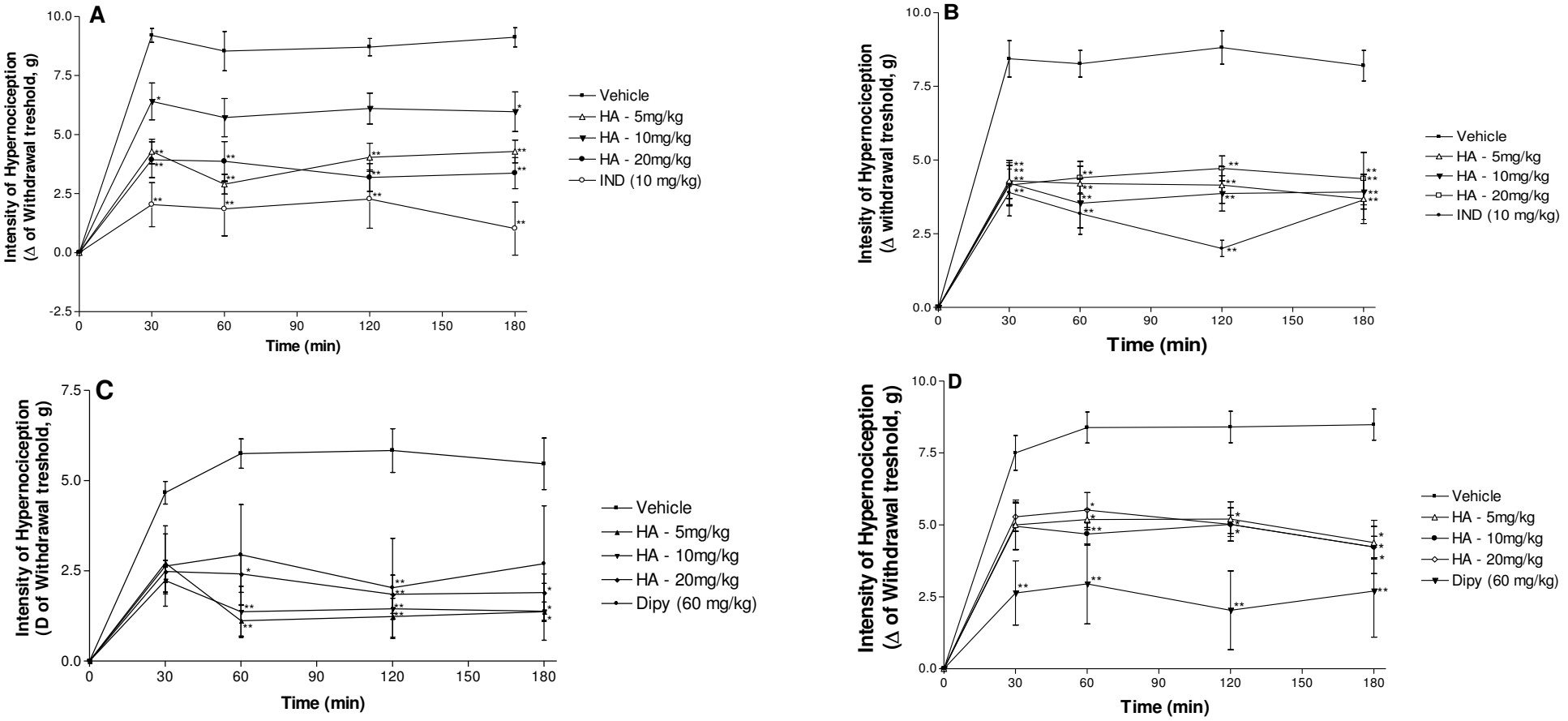

Figure 2. Effect of the acute administration of vehicle, hecogenin acetate (HA - 5, 10 or 20 mg/kg; i.p.) or reference drugs (indomethacin - 10 mg/kg; i.p. or dipyrone – 60 mg/kg, i.p.) on mechanical hyperalgesia induced by carrageenan (A), TNF-α (B), dopamine (C) or PGE_2 (D). Each point represents the mean ± SEM of the paw withdrawal threshold (in grams) to tactile stimulation of the ipsilateral hind paw. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus control group (ANOVA followed by Tukey test).

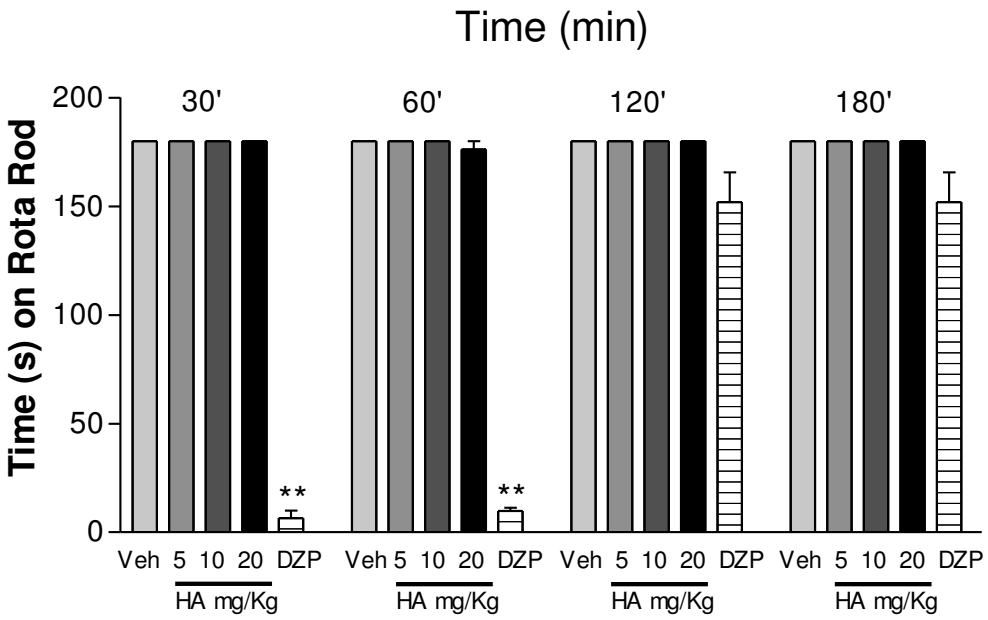

Figure 3. Time (s) on the Rota-rod observed in mice after i.p. treatment with vehicle (control), HA (5, 10 or 20 mg/kg) or diazepam (DZP, 1.5 mg/kg). The motor response was recorded for the following 180 s. after drug treatment. Statistical differences versus control group were calculated using ANOVA, followed by Tukey' test (n = 6, per group). *$p < 0.001$.

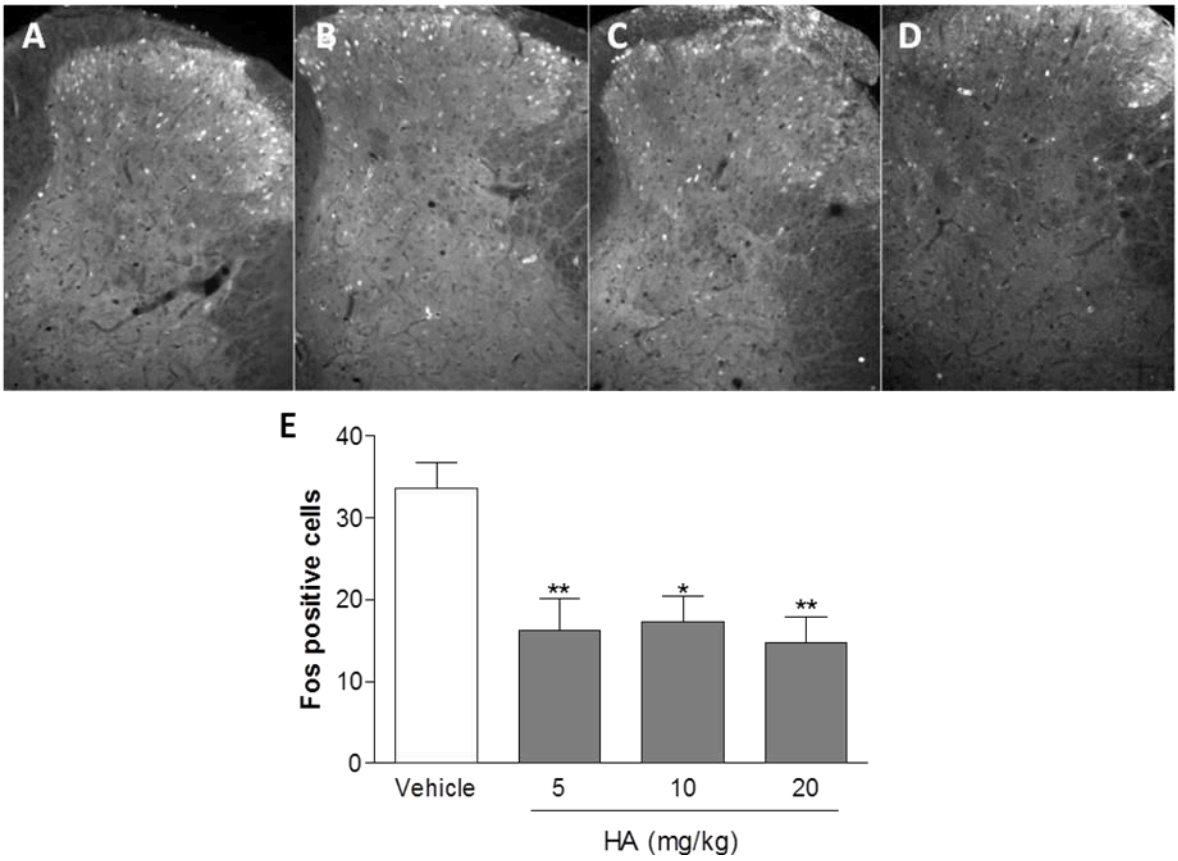

Figure 4. Immunofluorescence for Fos protein in the spinal cord dorsal horn, ninety minutes after the intraperitoneal injection of vehicle (A) hecogenin acetate at doses of 5 (B), 10 (C) and 20 (D) mg/Kg. The bar graph shows (below and right side) average FOS positive cells compared with the vehicle-treated group (white bar). Values represent mean ± S.E.M. (n = 6, per group). *$p < 0.05$, **$p < 0.01$ vs control (one-way ANOVA followed by Tukey's test).

4.3 CAPÍTULO 3

Short communication

Artigo a ser submetido ao BioMed Research International

Anti-hyperalgesic effect of hecogenin acetate on neuropathic pain model in mice

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Abstract

We used animal pain model to investigate the effects of hecogenin acetate (HA), a steroidal sapogenin-acetylated, on chronic neuropathic hypersensitivity (partial sciatic nerve ligation - PSNL) in mice. Acute treatment with HA (10 or 20 mg/kg, i.p.) was effective in producing a significanty ($p<0.01$) anti-hyperalgesic effect in PSNL model on mice. This effect no seems to be related to a possible myorelaxing profile of HA. So, our results indicates that HA might be potentially interesting in the study of new drug for the management of persistent pain.

Keywords: Hecogenin acetate, neuropathic pain, pain

Introduction

Neuropathic pain is caused by neuron injury in the peripheral or central nervous system (CNS). It can be caused by numerous pathological processes, e.g. multiple sclerosis, amputations, diabetes, shingles, and stroke. Opioids are less effective against this type of pain, but it may respond to anticonvulsants or tricyclic antidepressants [1]. However, pharmacological management remains the most important therapeutic option for chronic neuropathic pain, but results are still unsatisfactory and far from all patients obtain sufficient pain relief.

Natural products has been an invaluable source to obtaining new chemical entities with possible applicability in the treatment of painful conditions, including neuropathic pain [2]. Hecogenin acetate (HA, Figure 1) is a steroidal sapogenin-acetylated which it is an important precursors used by the pharmaceutical industry for the synthesis of steroid hormones. Recently, our group demonstrated that acute treatment with HA induced a pronounced antinociceptive profile involving opioid system and descending pain-inhibitory mechanisms. Now, we evaluate the antinociceptive effect of HA through neuropathic pain model in mice.

Material and Methods

Animals

Male Swiss mice (28-34 g), 2-3 months of age, were used throughout this study. The animals were housed in appropriate cages at 21 ± 2°C on a 12 h light/dark cycle (lights on 06:00-18:00 h) with free access to food (Purina®, Brazil) and water. Experimental protocols were approved by the Animal Care and Use Committee

(CEPA/UFS # 04/12) at the Federal University of Sergipe. All behavior experiments were performed under blind conditions to avoid influences of the observer in results.

Partial sciatic nerve ligation (PSNL)–induced neuropathic hypersensitivity

Mice were anesthetized by inhalation with oxygen 170 (3%) and isoflurane (2%). A partial ligation of the sciatic nerve was then performed by tying the distal one third to one half of the dorsal portion of the sciatic nerve with non absorbable 8-0 silk thread, according to the procedure previously described by Malmberg and Basbaum [3]. In sham-operated mice, the sciatic nerve was exposed using the same surgical procedure, but not ligated. The wound was closed with 4-0 silk thread suture and covered with iodine solution. Mice with ligated nerves did not present signs of paw clonus or autotomy. Mechanical hyperalgesia was evaluated by using digital Von Frey measurements (Insight®, Brazil), as described by Chaplan et al. [4]. Lesioned mice received once a day the HA (40 mg/kg, i.p.), gabapentin (70 mg/kg, i.p) or vehicle (10 mL/kg, i.p.) and sham-operated animals received only vehicle (10 mL/kg, i.p.) 7 days after surgery.

Grip strength test

Grip strength test was performed using a grip strength meter (Insight®, Brazil) as previously described by Meyer et al. [5]. The grip strength meter consists of a force transducer with digital display and a metal plate with a trapeze. Each mouse was placed on the plate and was pulled by its tail with increasing force until it was unable to grasp the trapeze and the grip was broken. The instrument digitally captures and displays the peak pull-force achieved. Muscle strength was defined as the peak weight (g) indicated on the display. Mice treatment was similar to the PSNL test.

Statistical analysis

The data obtained were evaluated by Two-way analysis of variance (ANOVA) followed by the Tukey test to compare the groups and doses over all times. In all cases differences were considered significant if $p < 0.05$.

Results and Discussion

Neuropathic pain is a complex disorder resulting from injury to peripheral nerve or certain areas of central nervous system (CNS), such as spinal cord or brain. The mechanisms of neuropathic pain are still incompletely understood and treatment is often unsatisfactory. So, the identification of novel therapeutic agents for the treatment of neuropathic pain is important. In the present study, we have investigated the anti-hyperalgesic effects of HA in neuropathic pain model in mice.

According to the results shown in Figure 2, the surgical procedure for inducing PSNL was effective, since the control group demonstrated a significant ($p<0.01$) increased in sensitivity to tactile stimulation in Von Frey apparatus, when compared with sham group. In addition, HA-treat mice a significant ($p<0.01$) anti-hyperalgesic effect during the first three hours of treatment, but this effect was reversed in the last valuation hour (6h). The reference drug, as expected, demonstrated striking anti-hyperalgesic effect.

Xu et al. [6] have reported that chronic pain induced by PSNL caused sustained dynorphin release that resulted in prolonged κ opioid receptor (KOR) activation in astroglial cells in the mouse spinal cord. Additionally, Wang et al. [7] have shown that dynorphin released in the spinal cord contributes strongly to the neuropathic pain response; thus, the modulation of pain-related areas on CNS, brain and spinal cord, involving opioid system appears to be one of the most important pharmacological

mechanisms to attenuate neuropathic pain on PSNL model. Recently, Gama et al. [8] has demonstrated that HA produced a consistent antinociceptive profile mediated by opioid receptors and endogenous analgesic mechanisms. Thus, It may be possible to suggest that CNS mechanisms may be involved in anti-hyperalgesic response induced by HA.

On the other hand, in order to clarify if the pharmacological effects of HA would be consequent to CNS activity interference on motor function, the activity HA was also evaluated on grip strength meter apparatus that it is a classical model for myorelaxant action providing information about psychomotor performance [9]. As shown in Figure 3 HA-treated mice did not cause any motor disturbance. This effect also corroborates that analgesic profile demonstrated by HA is a direct action in modulate pain by an understanding mechanism.

In conclusion, our study demonstrates that HA attenuates neuropathic pain without inducing loss in motor activity. In the light of the current clinical need for neuropathic pain treatment, this study provides novel evidence for additional therapeutic potential of HA.

Acknowledgments

This work was supported by grants from CNPq/Brazil (Grants # 551965/2010-0 and 472874/2012-8). We thank Abilio Borghi for the grammar review on the manuscript

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Figures

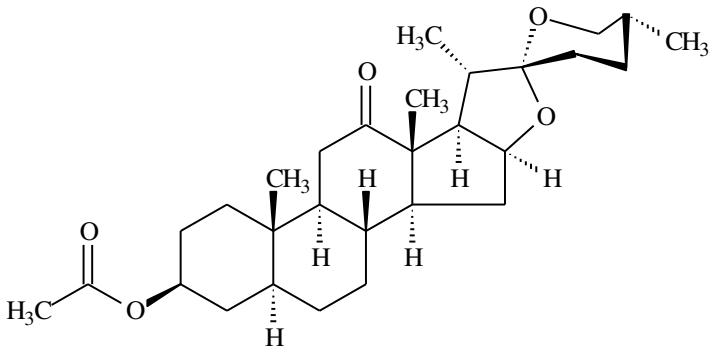

Figure 1 – Chemical structure of hecogenin acetate (HA)

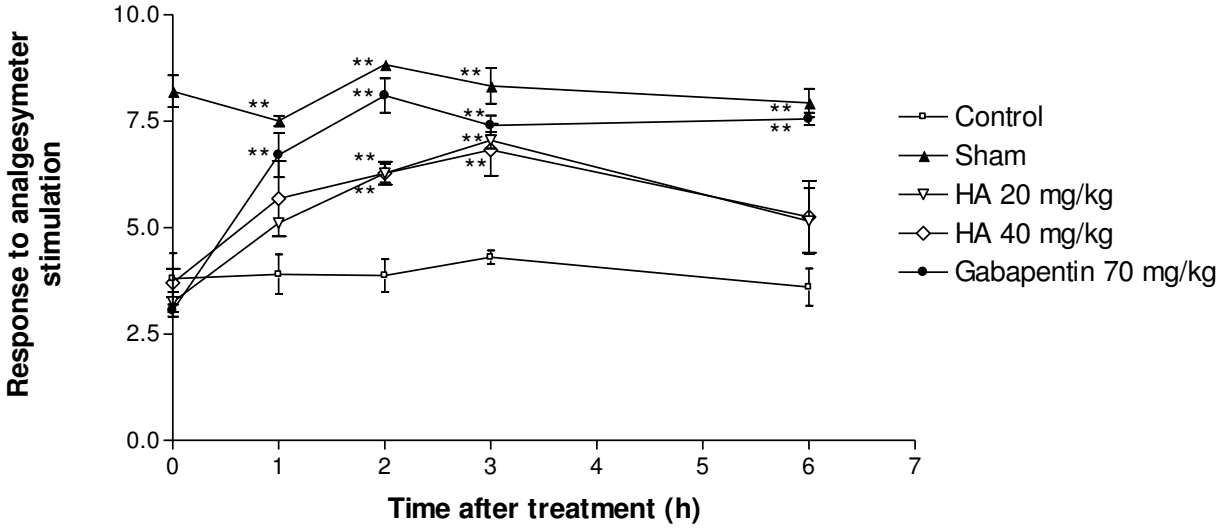

Figure 2. Effect of the acute administration of vehicle, hecogenin acetate (HA 10 or 20 mg/kg; i.p.) or reference drug (gabapentin - 70 mg/kg; i.p.) on mechanical hypernociception induced by PSNL. Each point represents the mean ± SEM of the paw withdrawal threshold (in grams) to tactile stimulation of the ipsilateral hind paw. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus control group (ANOVA followed by Tukey test).

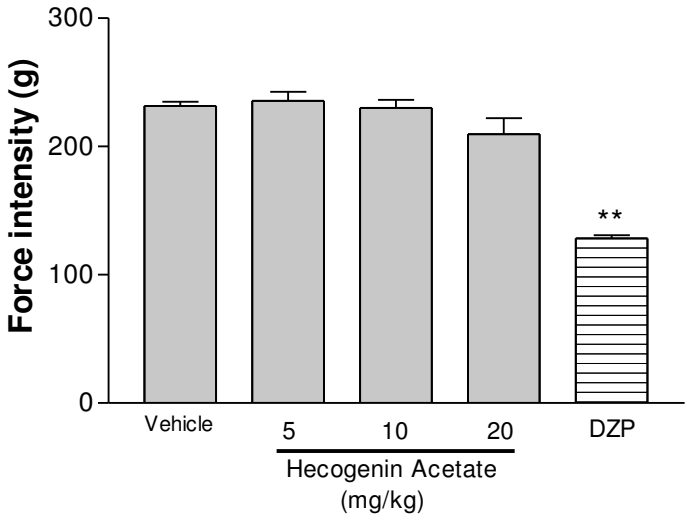

Figure 3. Effect of the acute administration of vehicle, hecogenin acetate (HA 5, 10 or 20 mg/kg; i.p.) or reference drug (diazepam - 10 mg/kg; i.p.) on grip strength test. Each point represents the mean ± SEM of the paw withdrawal threshold (in grams) to tactile stimulation of the ipsilateral hind paw. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus control group (ANOVA followed by Tukey test).

CONCLUSÕES

5. CONCLUSÕES

De acordo com os resultados obtidos no presente estudo podemos concluir que:

A administração sistêmica do acetato de hecogenina produziu em camundongos um pronunciado efeito antinociceptivo, com o possível envolvimento do sistema opióide e da via descendente da dor;

O acetato de hecogenina reduziu a nocicepção inflamatória e não inflamatória, estimulando neurônios da substância cinzenta periaqueductal e inibindo neurônios do corno dorsal da medula, desta forma corroborando com a hipótese do envolvimento da via descendente de controle da dor no perfil antinociceptivo;

O tratamento agudo com acetato de hecogenina reduziu o comportamento nociceptivo em modelo animal de neuropatia;

Os efeitos centrais do acetato de hecogenina, pelo menos nas doses testadas, não parecem estar relacionados com um possível efeito miorrelaxante ou neurotóxico em camundongos;

Os resultados são sugerem que o acetato de hecogenina pode ser uma molécula interessante para o estudo e desenvolvimento de preparações farmacêuticas com possível atividade analgésica.

PERSPECTIVAS

6. PERSPECTIVAS

O tratamento com acetato de hecogenina em camundongos mostrou-se efetivo em modelos de nocicepção em camundongos, sendo sugestivo seu efeito sobre o sistema opióide e, em especial, sobre a via descendente de controle da dor. Entretanto, como é comum no desenvolvimento de um projeto de doutorado novas perguntas surgiram a partir dos nossos resultados e que nortearão o grupo de pesquisa em Biotecnologia e Inovação Terapêutica em novas etapas deste estudo, a saber:

- Estudo da toxicidade aguda e crônica do acetato de hecogenina seguindo os critérios preconizados pela ANVISA (Agência Nacional de Vigilância Sanitária);
- Avaliação do possível envolvimento de outros sistemas de neurotransmissão no efeito antinociceptivo do acetato de hecogenina, tais como glutamatérgico, serotoninérgico e GABAérgico;
- Comparação dos efeitos antinociceptivo da hecogenina e do acetato de hecogenina;
- Avaliação de novas atividades farmacológicas, não avaliadas no presente estudo, por exemplo como atividade citotóxica, antioxidante, ansiolítica e gastroprotetora do acetato de hecogenina. Atividades essas já descritas para a hecogenina;
- Incorporação em sistemas de liberação controlada de fármacos avaliando seu possível emprego em protocolos crônicos de dor;
- Patenteamento e possível negociação com o setor produtivo do protótipo do invento, desde que seja assegurada sua eficácia e segurança terapêutica para posteriores estudos clínicos.

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ANEXOS

ANEXO A: Protocolo de Aprovação do Comitê de Ética em Pesquisa Animal da Universidade Federal de Sergipe (CEPA/UFS)

UNIVERSIDADE FEDERAL DE SERGIPE
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COORDENAÇÃO DE PESQUISA
COMITÊ DE ÉTICA EM PESQUISA COM ANIMAIS (CEPA)

DECLARAÇÃO

Declaro, para os devidos fins, que o Projeto de Pesquisa intitulado **"DESENVOLVIMENTO, CARACTERIZAÇÃO FÍSICO-QUÍMICA E AVALIAÇÃO DAS ATIVIDADES ANTINOCICEPTIVA E ANTIINFLAMATÓRIA DE NANOESTRUTURAS CONTENDO HECOGENINA COMPLEXADA EM B- CICLODEXTRINA"**, sob coordenação do **Prof. Dr. Lucindo José Quintans Júnior (protocolo CEPA 04/2012)** foi aprovado pelo Comitê de Ética em Pesquisa com Animais da Universidade Federal de Sergipe, "ad referendum".

São Cristóvão, 27 de abril de 2012.

Profª. Drª. Flávia Teixeira Silva
Presidente do CEPA/UFS

Cidade Universitária "Prof. Aloísio de Campos"
Jardim Rosa Elze – São Cristóvão – SE
49100-000
Fones: 3212 6661/6606

ANEXO B – Trabalhos apresentados em congressos relacionados ao tema da tese.

Resumo apresentado no Experimental Biology – 2012, San Diego, CA, USA. Os resumos foram publicados num número especial do The FASEB Journal.

(*The FASEB Journal*. 2012;26:662.3)

662.3

HECOGENIN REDUCES HYPERNOCICEPTION IN MICE

Jullyana Siqueira Quintans[1], Angelo Antoniolli[2], Makson GB Oliveira[4], Michele F Santana[3], Valter J Santana-Filho[2], Alexsandro Branco[5], Jackson RGS Almeida[6], Alex G Taranto[7], Rosana SS Barreto[2] and Lucindo J Quintans, Junior[2]

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The present study examined the anti-hypernociceptive effect of the hecogenin (HG) in mice. For assess this effect were realized models of mechanic hypernociception induced by carrageenan (CG) and Tumor Necrosis Factor-α (TNF-α). The mice were pre-treated with vehicle (saline + Tween 80 0.2%; p.o.) or HG (5, 10 and 20 mg/kg, i.p.). Thirty minutes after the treatments, were injected in region subplantar of the rear right paw: 20 µL of CG (300 µg/paw) or TNF-α (100 pg/paw). The hypernociception was assessed in times of 0.5, 1, 2 and 3 hours after the injection of CG and TNF-α, with digital Von Frey. It was observed that HG was able to maintain the baseline nociceptive threshold in two tests. The results suggest anti-hypernociceptive property of HGN, probably by a mechanism inhibition of production of cytokines, which activate various reactions connected with inflammation, reducing thereby hipernociception.

Financial support: FAPITEC/SE, CAPES, CNPq (Brazil).

Resumo apresentado no Experimental Biology – 2013, Boston, MA, USA. Os resumos foram publicados num número especial do The FASEB Journal.

(*The FASEB Journal*. 2013;27:lb524)

lb524

c-Fos expression in the piriform cortex and periaqueductal gray after hecogenin acetate administration on carrageenan-induced hypernociception test

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We evaluate the c-Fos expression in the mice central nervous system areas (CNS), piriform cortex and periaqueductal gray (PAG), after acute administration of hecogenin acetate (HC) on carrageenan-induced nociception test. So, carrageenan-induced hypernociception model was performed in mice as reported by Guimarães et al. (2012); c-Fos analysis was held according to Brito et al. (2013) and grip strength test according to Meyer et al. (1979. Protocols were approved by the Animal Care and Use Committee at the UFS (CEPA/UFS # 04/12). Ninety minutes after the HC intraperitoneal injections (5, 10, or 20 mg/kg), the mice submitted to hypernociception induced by carrageenan (300 µg/paw), the average number of neurons showing Fos protein was increased in PAG area and only in higher dose produced increase in olfatory bulb area. HC not produced significant alterations in piriform cortex area. Interestingly, systemic administration of HC, in all tested doses, produced an anti-hypernociceptive effect in this model. So, such results were unlikely to be provoked by motor abnormality when evaluated in grip strength test. Our results provide evidence to propose that HC produced a lack activation of olfatory bulb, but induced a strong PAG-activation, at the all tested doses, indicates a possible involvement in descending pathways that modulate pain.

ANEXO C – Normas do Jornal Phytomedicine para submissão do artigo: **Natural products evaluated in neuropathic pain models – a systematic review**

Phytomedicine
International Journal of Phytotherapy and Phytopharmacology

Instructions to Authors

Aims and Scope

The following areas are covered:

Clinical, pharmacological, molecular/genomic, pharmacokinetic and bioavailability studies of standardized plant extracts, fractions, isolated constituents and phytopharmaceuticals thereof having significant bioactivities or could be promising candidates for further thorough pharmacological and clinical studies.

1. Basic and stringent Requirements for consideration of submitted papers:

The standardization of all above listed plant materials used for the investigations, has to be carried out by means of HPLC, HPLC/MS or HPLC/NMR fingerprinting; inclusive the identification and quantitation of the main bioactive compounds which are or might be responsible for pharmacological activities. The methods have to be described in details: apparatus, columns, solvent systems, gradient, flow rate, detection etc. If the authors do not possess the required analytical equipment or expertise, they are asked to seek cooperation with a phytochemical laboratory. For all plant materials used in investigations stated as derived from cultivated plants or from their natural origin, voucher specimens must be deposited in a specific location with a voucher number. The site (GPS coordinates) and date of collection, with the part(s) used in the study, have to be documented.

Without phytochemical standardisation of the plant extracts, the results presented cannot be pharmacologically reproduced and are not acceptable for experimental and clinical studies.

Note: With immediate effect Phytomedicine will only accept two revisions of a manuscript.

2. The following areas have a restricted scope within Phytomedicine

- Papers on the isolation and structure elucidation of novel bioactive compounds or the development of new analytical methods do not fall into the scope of Phytomedicine and should be reported elsewhere (e.g. Phytochemistry, Journal of Chromatography or Phytochemical Analysis). Extraordinary pharmacological and clinical studies of these novel natural products, however, are welcome.
- Screening results of a large number of plant extracts or plant constituents for antimicrobial or other pharmacological activities will not be considered unless they are focused on those plants or constituents which show extraordinary activities in comparison with internationally accepted positive (reference) compounds.
- "Dietary Supplements", "Botanicals" or "Functional Food" are not within the scope of Phytomedicine unless they are standardized and pharmacologically investigated analogue to herbal drugs and if the evidences presented are comparable to therapeutic outcomes of a positive control.

Clinical Studies

- Clinical studies must be designed, implemented and analyzed in a manner to meet current standards for clinical trials (GCP = Good Clinical Practice), which are equivalent to those required for synthetic drugs.
- For guidelines and necessary information see the following internet address: www.consort-statement.org with the "Revised Recommendations for Improving the Quality of Reports of Parallel-Group Randomized Trials" which provides links for downloading the Consort Statement and a checklist as well as explanatory and elaboratory documents. Extensions of the Consort Statement for different types of trials including Herbal Medical Interventions are provided. (The Consort Statement is available in 10 different languages).
- Clinical studies must be approved by an Institutional Ethics Committee or its equivalent and it must be stated in the Method section that the research followed the guidelines of the Declaration of Helsinki and Tokyo for humans.

Pharmacological and molecular biological studies (*in vitro*, *ex vivo* or *in vivo*)

- Investigations with animals must state in the method section that the research was conducted in accordance with the internationally accepted principles for laboratory animal use and care with stating the guidelines (e.g. European community guidelines/ EEC Directive of 1986 or the US guidelines/ NIH publication).
- Results have to be based on adequate statistics. Positive controls (reference/

standard compounds) and at least three dose responses for conventional pharmacological experiments have to be included.

- Many polyphenolic- and terpenoids containing plant extracts exhibit polyvalent (pleiotropic) activities. Such extracts are of interest for further thorough pharmacological and therapeutic investigations only if one or two pharmacological activities are dominant and justify the therapeutic application for specified indications.
- Pharmacological studies with herbal drug combinations (e.g. 2–5 plants) will be accepted only if the single herbal extracts are HPLC-finger printed and their major bioactive constituents are quantified before the single extracts are mixed (combined) (see also as an example for the 3D-HPLC-analysis of multidrug combinations Annagaya S. et al., 2008, Phytomedicine 8, 338–342.)
- Two plant extracts or a single constituent of these combined with a synthetic drug or antibiotic which are suggested to exhibit synergistic effects have to be investigated by the "isobol method" according to Berenbaum M. 1989, Pharmacol. Rev. 41: 93–141. (see also Wagner H. and Ulrich-Merzenich G. Synergy research: Approaching a new generation of phyto-Pharmaceuticals Phytomedicine 16: 97–110 (2009).
- Antimicrobial evaluation of plants are of scientific value only if these plant extracts show extraordinary biological activities in comparison with a synthetic or natural antimicrobial agent standard. It is not useful if the in vitro activity (MIC) of an extract exceeds 100µg/ml. For the correct determination of MIC values, see Eloff J.N., 2008, Phytomedicine 11: 3701.
- Papers which describe classes of pharmacological activities such as flavonoids with antioxidative activity and isoflavones with estrogenic antiinflammatory activity, will be accepted only if the activities presented exceed those of standard substances and could be promising candidates for further pharmacological and clinical investigations.
- All papers reporting gene expression profiling data
- (microarray experiments) should comply with the Minimum Information about microarray experiments (MIAME) standard: www.mged.org/Workgroups/MIAME/miame.html.

At least two **microarrays** should be provided for each experimental condition. Results of selected genes should be validated by a second method (e.g. RT-PCR) or protein data should be provided. In addition functional test (animal experiments/clinical data) undertaken simultaneously are desirable to allow an appraisement of the biological/clinical relevance of the data. Alternatively, results of in vivo experiments with comparable dosages can be discussed. The presentation of a sole data collection is not acceptable. Biologically relevant information should be presented.

Gene nomenclature

Authors should use approved nomenclature for gene symbols. Please consult the appropriate nomenclature data bases for correct gene names and symbols. "Entrez Gene" is a useful resource. Approved human gene symbols are provided by HUGO Gene Nomenclature committee (HGNC): www.gene.ucl.ac.uk/nomenclature. Approved Mouse symbols are provided by The Jackson Laboratory: www.informatics.jax.org/mgihome/nomen. Approved C. elegans symbols are provided by Caenorhabditis Genetics Center: http://www.cbs.umn.edu/CGC/Nomenclature/nomenclat.htm. For approved S. cerevisiae and S.pombe Symbols see http://seattleanome.org/help/yeastGeneNomenclature.html and respectively: www.sanger.ac.uk/Projects/S_pombe/SP_Name_FAQ.shtml.

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Prevention of Plagiarism

Contributions are accepted on the understanding that the authors have obtained the necessary authority for publication. Submission of multi-authored manuscripts implies the consent of each

of the authors. The publisher will assume that the senior or corresponding author has specifically obtained the approval of all other co-authors to submit the article to this journal. Submission of an article is understood to imply that it is not being considered for publication elsewhere and that the author(s) permission to publish his/her article in this journal implies the exclusive authorization to the publisher to deal with all issues concerning copyright therein. Further information on copyright can be found on the Elsevier website.

Preparation and Format of manuscripts

Manuscripts submitted to Phytomedicine should be structured in the following manner:

Title: Full **author names** referenced by arabic superscripts with affiliation and addresses of all authors, e.g. A. Hymete[a], T.H. Iversen[a], J. Rohloff[a], B. Erko[b]

[a]Department of Biology, The Plant Biocentre, Norwegian University of Science and Technology N-7491 Trondheim, Norway
[b]Institute of Pathobiology, Addis Ababa University P.O. 1176, Addis Ababa, Ethiopia
*The phone, fax and email address of the corresponding author should be placed on the cover page.

An **Abstract** should contain brief information on purpose, methods, results and conclusions in no more than 1000 words.

Keywords: Not more than six words

A section of **abbreviations** should precede the manuscript with molecular biological content (see also section "microarray data")

Introduction

Materials and Methods

Results

Discussion

A combined **Results and Discussion section** may also be appropriate.

Acknowledgement

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Liu, C.D., Kwan, D., Saxton, R.E., McFadden, D.W., 2000. Hypericin and photodynamic therapy decreases human pancreatic cancer in vitro and in vivo. J. Surgical Res. 93, 137–143.

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ANEXO D – Confirmação de submissão do artigo: **Antinociceptive activity of hecogenin acetate, a steroidal sapogenin-acetylated, in experimental inflammatory pain: a possible involvement of the spinal cord inhibition**

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ANEXO E – Normas do Journal of Pharmacy and Pharmacology, para submissão do artigo: **Antinociceptive activity of hecogenin acetate, a steroidal sapogenin-acetylated, in experimental inflammatory pain: a possible involvement of the spinal cord inhibition**

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One author: Szeto HH. Simultaneous determination of meperidine and normeperidine in biofluids. J Chromatogr 1976; 125: 503–510.

Two authors: Vu-Duc T, Vernay A. Simultaneous detection and quantitation of O6-monoacetylmorphine, morphine and codeine in urine by gas chromatography with nitrogen specific and/or flame ionization detection. Biomed Chromatogr 1990; 4(2): 65–69.

Three or more authors: Huestis MA et al. Monitoring opiate use in substance abuse treatment patients with sweat and urine drug testing. J Anal Toxicol 2000; 4(Suppl.3): 509–521.

Article in press: Ladines CA et al. Impaired renal D1-like and D2-like dopamine receptor interaction in the spontaneously hypertensive rat. Am J Physiol Regul Integr Comp Physiol 2008 (in press).

Electronic publication ahead of print: Teeuwen PHE. Doppler-guided intra-operative fluid management during major abdominal surgery: a systematic review and meta-analysis. Int J Clin Pract (accessed 21 November 2007, epub ahead of print).

Online serial: Margolis PA et al. From concept to application: the impact of a community-wide intervention to improve the delivery of preventive services to children. Pediatrics [online] 2001; 108:e42. www.pediatrics.org/cgi/content/full/108/3/e42 (accessed 20 September 2001).

Corporate author: The Cardiac Society of Australia and New Zealand . Clinical exercise stress testing. Safety and performance guidelines. Med J Aust 1996; 164: 282–284.

Anonymous author: Anon. Coffee drinking and cancer of the pancreas. BMJ 1981; 283: 628.

Author with prefix and/or suffix in their name: Humphreys Jnr, Sir Robert and Adams T. Reference style in the modern age. J Bib Cit 2008; 1: 1–10.

Article not in English: Sokolov S et al. [Studies of neurotropic activity of new compounds isolated from Rhodiola roseaL.] Khim Farm Zh 1985; 19: 1367–1371 [in Russian].

Book references

Book by a single author or group of authors working together as a single author: Cole MD, Caddy B. The Analysis of Drugs of Abuse: An instruction manual, 2nd edn. New York : Ellis Horwood, 1995.

An edited book:Hoepfner E et al. eds. Fiedler Encyclopedia of Excipients for Pharmaceuticals, Cosmetics and Related Areas, 5th edn. Aulendorf: Editio Cantor Verlag, 2002.

An article in an edited book:Sanders PA. Aerosol packaging of pharmaceuticals. In: Banker GS, Rhodes CT , eds. Modern Pharmaceutics. New York : Marcel Dekker, 1979: 591–626.

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ANEXO F – Normas do Jornal BioMed Research International para submissão do artigo: **Anti-hyperalgesic effect of hecogenin acetate on neuropathic pain model in mice**

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