

**Evaluation of the Antinociceptive Properties of
Hyptis crenata Pohl (Brazilian Mint)**

Thesis submitted in partial fulfilment of the requirements for the degree of
Doctor of Philosophy

By

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Abstract

This project aimed to investigate the main traditional use of the plant *Hyptis crenata* (HC) and evaluate its activity in a pre-clinical trial. A traditional use survey was carried out in two municipalities in Brazil, interviewing 20 people. The results showed that the main use of HC is for pain relief (19/20). The main method used for its preparation was decoction extract (11/20). The antinociceptive activity of HC decoction extract was evaluated showing that HC 15 mg/kg (p.o.) and HC 150 mg/kg (p.o.) increased delay in withdrawal response in the Hargreaves thermal withdrawal test by 29% and 28% respectively and decreased writhing occurrences induced by acetic acid by 70% and 71% ($p < 0.05$), when compared to vehicle. These treatments were examined in the acetic acid writhing test for their effects on c-fos protein expression. The results showed that HC 150 mg/kg (p.o.) decreased c-fos expression in the hypothalamic paraventricular nucleus by 40% ($p < 0.05$). This study also evaluated the HC doses 1 mg/kg (p.o.), 5 mg/kg (p.o.) and 45 mg/kg (p.o.) to assess the dose-response effect. HC dose-dependently induced antinociception in both animal models from 0 mg/kg (p.o.) to 15 mg/kg (p.o.), until a plateau response occurred between 15 mg/kg and 45 mg/kg (thermal withdrawal test $p = 0.0002$ and writhing inhibition $p = 0.4725$). Then, the decoction extract was fractionated and tested in an attempt to identify which HC compounds were active in inducing the antinociceptive effect. The hexane fraction had the highest activity (per dose) compared to the other fractions, indicating that it was enriched with the active compounds. Also, a preliminary COX inhibition assay was carried out; the results indicating possible COX-2 inhibition for HC treatments. Additionally, an investigation through behavioural analyses of whether HC and its fractions were affecting basal behaviour, such as muscle relaxation and sedation, showed no change of such behaviour. Overall, these data support the antinociceptive effect of *Hyptis crenata* and that there are specific compounds responsible for this effect.

Declaration

I certify that this thesis is the result of my own investigations and that no part of it has been submitted for any degree other than the Doctor of Philosophy at the University of Newcastle upon Tyne. All references to the work of others are acknowledged.

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Abbreviations

B.C.	Before Christ
c-fos	Immediate early gene family of transcription factors
CNS	Central nervous system
COX	Cyclo-oxygenase enzyme
DAPI	4',6-diamidino-2-phenylindole
DCM	Dichloromethane
DLPAG	Dorsal lateral periaqueductal grey
DMPAG	Dorsomedial periaqueductal grey
FITC	Fluorescein
GABA	Gamma-aminobutyric acid
GC-MS	Gas chromatography mass spectrometry
GPCR	G-protein-coupled receptors
HC	<i>Hyptis crenata</i>
HF	<i>Hyptis fruticosa</i>
HP	<i>Hyptis pectinata</i>
HPLC	High-performance liquid chromatography
HS	<i>Hyptis suaveolens</i>
ID	Indomethacin
LC-MS	Liquid chromatography-mass spectrometry
LPA	Lysophosphatidic acid
mRNA	Messenger RNA

PAG	Periaqueductal grey
PBS	Phosphate-Buffered Saline
PFA	Paraformaldehyde
PGE ₁	Prostaglandin E1
PGE ₂	Prostaglandin E2
PVA	Paraventricular thalamic nucleus, anterior
PVN	Paraventricular nucleus of the hypothalamus
R.T.	Retention time
SA	Salicylic acid
SO	Supraoptic nucleus
THC	Tetrahydrocannabinol
VLPAG	Ventrolateral periaqueductal grey

n= Number

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Chapter 1. Introduction and Literature Review

1.1 Traditional Use of Medicinal Plants

1.1.1 Historical background of herbal medicine

The use of medicinal plants is not novel, in fact this form of medication has been used by humans for thousands of years. There are fossil records showing that since the middle Palaeolithic age at least 60,000 years ago plants were used for medicinal purposes (Leroi Gourhan, 1975). This timeline can also be affirmed by the discovery of a Neanderthal archaeological site in Iraq dated to 60,000 B.C. that revealed pollen of several species of medicinal plants being used (Gurib-Fakim, 2006).

In relation to the history of herbal medicine, its origin and development, there are some distinctions involving continents, countries and cultures. For instance, African, Greek, Indian and Chinese each have distinct histories. The African continent is described as being the oldest and perhaps the most diverse application of medication, including phytotherapy. In the African continent, traditional medicines have varied forms, usually based on a holistic concept involving body and mind. The diagnoses made by the healer are for treatment of the psychological basis first and after medicine is prescribed to treat the symptoms (Gurib-Fakim, 2006). Also, in countries like South Africa, the traditional healer, known as Dingaka or Invanga, believed that preparations involving plant, animal and insect parts produce a powerful synergistic action for treatments (Louw *et al.*, 2002).

The ancient Greeks also played an important role in introducing phytotherapy as medicine, but this system incorporated a close relation between their Gods and the plant. As a result, some plants that are known today, such as *Origanum dictamnus* and *Artemisa absinthium*, were first named in dedication to the goddess Artemis-Diktyinna a thousand years B.C. (Liolios *et al.*, 2010). This “god-plant belief” together with Greek medicine were drastically decreased after the end of the Roman Empire due to the fact that this knowledge was not valued by the Christian Byzantines (Lardos, 2006). However, some of this ancient knowledge survived, and can be found in the classical documents written by Hippocrates (5th century B.C.), Dioscorides (1st century A.D.), and Galen (2nd century A.D.) (Karousou and Deirmentzoglou, 2011).

Another nation that has made an important contribution to phytotherapy is India. This country has a very ancient practice of traditional medicine named Ayurveda. Unlike the ancient Greek, this medicine is still being practiced in India (Dwivedi, 2007). There are

many plants reported for their therapeutic properties in the Ayurveda system and one of the oldest documents, dated to 100-500 B.C., describes 700 plants with descriptions of their therapeutic properties (Patwardhan, 2005; Patwardhan *et al.*, 2005).

Like Ayurveda, traditional Chinese medicine is also an ancient system of treatment and is dated to more than 5000 years old (Gurib-Fakim, 2006). It is distinguished from medicine which focuses on individual treatment, such as Western medicines, by the concept of traditional Chinese medicine that uses plants that will adjust the balance of the body, thereby improving its pathology (Xiang *et al.*, 2008). This medical system has been disseminated to other Asian countries, including Japan where nowadays it is known as Kampo medicine (Ushiroyama, 2005).

The various systems of medicine used by these cultures have been distributed and have spread to other continents when the process of colonisation took place. The outcome of this process was a fusion of knowledge between the coloniser and original inhabitant of the territory. An example of this applies to countries colonised in Central and South America. Using Brazil as an example, this country has a vast biodiversity of plant species that were used as medicine by the natives. Although the traditional indigenous use of herbs has predominated, the herbal medicine systems have undergone modification due to the process of colonisation carried out by Europeans that used the Western herbalism system that originated in Greece and Rome, and also the introduction of Africans during the slavery period (Monteiro *et al.*, 2010; De Melo *et al.*, 2011).

This is the historical background of medicinal plants, but it does not mean that use of medicinal plants belongs to the past. In fact, it is still in use worldwide and its demand has been progressively increasing in recent decades in the form of phytotherapy. The next section will describe the traditional medicine that is still in use and the commercialisation of phytotherapeutics.

1.1.2 Current use of plants as medication

As mentioned, some of the traditional medicine systems have contributed to the origin of different types of medicine that are still being used worldwide. They can be classified into four basic systems: (i) traditional Chinese herbalism or Kampo medicine in Japan;

(ii) traditional Indian medicine, Ayurvedic herbalism; (iii) Western herbalism; and (iv) traditional African, Arabic and Islamic medicine (Azaizeh *et al.*, 2008).

The traditional Chinese herbal medicine lists more than 11,228 plant species. This fact makes it the most comprehensive form of herbalism worldwide (Sucher, 2006). It has recently been reported that there are more than 600 plants being used by the Chinese pharmacopoeia (Zheng *et al.*, 2011).

With respect to Ayurvedic medicine, in India the use of this medicinal system provides healthcare to about 80% of the population in India, i.e. around 1 billion people (Jain *et al.*, 2005; Tripathi *et al.*, 2011).

Ethnobotanical surveys from various places in Europe that use Western herbalism have shown that it is still widely practiced in these regions (Azaizeh *et al.*, 2008). In Cyprus, for example, there are shops selling a variety of herbs that are used mainly for neuropsychiatric, gastrointestinal, and respiratory ailments (Karousou and Deirmentzoglou, 2011). In Navarra, in the Iberian Peninsula, with a population of 144,674 inhabitants, the use of 90 medicinal plants belonging to 39 botanical families is reported. These species include *Santolina squarrosa*, *Thymus vulgaris*, *Rosmarinus officinalis* and *Urtica dioica* (Calvo *et al.*, 2011). A systematic review of the traditional use of wild and cultivated plants in north-eastern Bosnia and Herzegovina, Southeast Europe, showed the use of 254 plant species with 1655 different preparation methods. The most frequently mentioned indications were disorders of the gastrointestinal tract, respiratory system, urogenital tract, skin, blood system, cardiovascular system, nervous system and rheumatism (Saric-Kundalic *et al.*, 2011).

Herbalism that has spread to or from North and South America is classified as Neo-Western herbalism. As mentioned earlier this medicine system applies formulations prescribed in ancient pharmacopoeias from western medicines and also preparation methods indicated by indigenous origin. An example of this system is the use of the plant *Echinacea* as an immune stimulant for the treatment of colds (Elvin-Lewis, 2001). The Tehuacán-Cuicatlán Valley, in central Mexico, is reported to have 2800 plant species with 88 of these species used in traditional medicine (Pérez-Negrón and Casas, 2007). In Latin America, social transmission of medicinal plant knowledge and skills is passed on by an experienced healer or elderly relatives, and some of them, due to their

spiritual beliefs, claim that the information is acquired through dreams (Vandebroek *et al.*, 2004).

As described above, the use of plants in Brazil has been influenced by its cultural mixing. However, another factor that plays an important role is the geographical conditions that will make possible for certain species to be available. Therefore, in Brazil the traditional use of medicinal plants can be divided into five categories according to its vegetation distribution and cultural background. These are North (Amazon Forest); Northeast (tropical forest and Caatinga which is a desert-like terrain); Southeast (Atlantic forest and Cerrado that is savannah-like vegetation); Central-West (Cerrado and Pantanal); and South (rainforest along the coast known as Mata Atlântica, tropical semi-deciduous forest known as Araucárias, and Pampas). The size of the area and the variety of different species caused by geographical variability contributes to the number of plant species being used. For instance, an ethnobotanical survey in the central west area showed that many species were being used in comparison with the relatively small number of people interviewed. As reported in the survey carried out in the Monjolinho settlement in Mato Grosso do Sul state that shows 210 different species being used by 35 people (da Cunha and Bortolotto, 2011). On the other hand a survey involving 92 people from Rio Grande do Norte in the Northeast region shows only 45 species being used (Silva and Freire, 2010).

In summary, these reports show that the concept of using medicinal plants as a form of treatment is an ancient knowledge that is still spreading worldwide. However the method of use and the plant species varies according to cultural belief, type of treatment needed and geographical influences that determine which species would be available to be used.

1.2 Research on Medicinal Plants

1.2.1 Pain relief drugs developed based on medicinal plants

There are several medicinal plant compounds that have been utilised in pain relief drug discovery. Perhaps two of the most well-known would be morphine and salicylic acid. The former was isolated by a German pharmacist Friedrich Serturmer in 1804. He named the compound and defined its class as an alkaloid (Klockgether-Radke, 2002). Morphine was extracted from the poppy *Papaver somniferum* and since its discovery it

has been used for pain therapy and currently is particularly used against cancer pain (Cherny, 2000). Salicylic acid was isolated from the white willow *Salix alba* and later synthesised as acetylsalicylic acid by the pharmaceutical company Bayer, which named it “Aspirin”, the change in the chemical structure enabled this drug to become more palatable (Mackowiak, 2000). In 1971 the pharmacologist John Vane studying the effects of aspirin identified that its mechanism involved inhibition of prostaglandins. He also suggested that the salicylate was more effective in inhibiting PGE₁ than PGE₂ (Vane, 1971). Further research investigating the pain relief mechanism of morphine and salicylic acid led to the identification of the opioid receptors and the understanding of cyclooxygenase inhibition mechanisms that will be discussed later in this chapter.

After Bayer’s work with salicylic acid other pharmaceutical companies, like Merck, also turned their attention towards chemical compounds from plants, and started screening plants used by traditional systems like the Chinese. However the aim was to find compounds that could be synthesised and modified into a drug in the same way as acetylsalicylic acid (Borris, 1996).

In the course of time a different approach has been adopted in the search of compounds from medicinal plants, due to the high cost of compound synthesis and also because more people started looking for more natural forms of medication. Complementary and alternative medicines (CAM) became popular, using herbal therapy that has its focus on using the entire extract rather than a single compound (Desmarchelier, 2008; Zareba, 2009). According to WHO the commercialisation of these products has generated income of US\$ 5 billion in 2003-2004 for western societies and for China US\$ 14 billion in 2005. Another explanation for this increasing demand could be that herbal medicine represents a valuable resource in disease prevention rather than treatment, this is the case for therapy of diseases affecting the central nervous system, such as chronic neurodegenerative disorders, e.g. Alzheimer's disease and Parkinson's disease (Iriti *et al.*, 2010).

In general, the compounds from medicinal plants have contributed greatly to the discovery and development of important drugs that have played crucial roles for human health. An example of this is the antimalarial agent artemisinin, which was extracted from sweet wormwood, *Artemisia annua*, and has been manufactured by many companies, including Merck and Bayer (Borris, 1996). The search for plant compounds still continues and one major focus for decades has been to find an efficient treatment

for cancer. The National Cancer Institute in United States launched a large project in 1960 for screening anti-tumour agents from medicinal plant compounds, they evaluated 35,000 plant species. As a result of this program, the compound taxol from the yew tree was identified and so far it has been very successful as an anti-tumour drug (Balunas and Kinghorn, 2005; Ghantous *et al.*, 2010).

1.2.2 *The process of evaluation of plant activities and their pharmacological properties*

Many research groups have been evaluating medicinal plants with a history of traditional use and their compounds as a source of new clinically useful medicines (Srinivasan, 2005). However, there are concerns and criticisms regarding the reliability of results from much of the research on medicinal plants, mainly related to the quality and appropriateness of the methods used (Wirth *et al.*, 2005; Chan and Chan, 2008). This would also apply for studies evaluating pain relief properties.

When evaluating antinociceptive effects in animals it is important to use the right pain assessment methods and to check for false positive results that can occur, for example when muscular relaxation is interpreted as antinociception (Le Bars *et al.*, 2001). However, studies of the effects of medicinal plants on pain in animal models differ conceptually from the use of the same models as part of the development of synthetic antinociceptives, and a failure to take this into account when developing the experimental protocols may render the results of the research irrelevant for either purpose.

When evaluating medicinal plants in particular, any available information about traditional use of the plant species should be considered when deciding the methods of preparation and dosage (Fabricant *et al.*, 2001). Testing a synthetic compound for pain relief in an animal model usually takes place at a stage where the mechanism is already known and the aim is to investigate dosages and toxicology of the compound. In contrast, in a medicinal plant evaluation the relevant doses can usually be estimated from information about the traditional use, while there is a need to evaluate the magnitude of the effects (if any) and assess which mechanisms may be involved.

It is important to investigate the traditional use of medicinal plants to create a database and guidelines for methods of use and preparations, also such information could be used

to develop new drugs and contribute to medical treatments. In relation to traditional use of any medicinal plant, its long term use by humans gives some advantages when using its lead compounds to develop a new drug, due to the fact that their bioactive compounds have been used for many generations. Furthermore, as a result of their long term use it can be assumed that they are more likely to have a low toxicity for humans. Unfortunately, literature on medicinal plants used in traditional medicine usually do not report detail on the therapeutic uses and method of preparation (Chinsamy *et al.*, 2011).

There are relatively few studies that have evaluated through pharmacological trials the information obtained from a traditional use survey. Usually the pharmacological evaluation is made by other research groups. Furthermore, there are few traditional use studies that describe with detail the preparation methods for the plants mentioned. The majority of studies which have carried out surveys and performed pharmacological studies afterwards are the studies involving antiplasmodial activity (Sanon *et al.*, 2003). Although this fact gives an impression that there is little integration between ethnobotanical and pharmacology areas, this might be a misleading concept. Some research groups that reported ethnobotanical studies might also be carrying out pharmacological trials, but publishing in separate documents.

1.3 Pain

The word "pain" was originated from the Latin "*poena*", meaning a fine or a penalty (MedicineNet, 2010). As the word suggests it is a very unpleasant sensation but it is also crucial for the protection of the body and for the process of healing damaged tissues. However, pain can become chronic and abnormal, through pathophysiological processes causing problems not only for the patient but consequently for the public in general due to the total cost of pain for the national economy (Woolf and Salter, 2000). Reports from the British Pain Society show that in the United Kingdom millions of working days are lost due to pain caused by arthritis, and an large sum of money, £5 billion is spent per annum to treat pain symptoms such as back pain (British Pain Society 2008). This type of pain or any other chronic pain still represents difficult challenges for medicine because in many of these cases there is no efficient treatment available. Therefore, the patients need to adapt to live with the problem for the rest of their lives. (Melzack and Wall, 1996).

1.3.1 Describing and classifying pain – Acute and chronic pain

Although sense of pain has an established physiological basis, the sensation varies according to individual experience. The major classification of pain is according to its duration and the type of trigger that will transform into the pain sensation. The term acute pain is used for pain that lasts only for a relatively short period (hours-days). The purpose of this type of pain is to induce the individual to react and protect the part of the body injured until it is healed. After it is healed, the pain should disappear (Melzack and Wall, 1996). However in some cases the patient still suffers the pain sensation even after the original injury has healed and for this reason it becomes classified as chronic pain. This is an incessant noxious stimulus sensation that can be prolonged from months to years and is provoked by a dysfunction of the physiological pathway (Cheville *et al.*, 2000). This type of sensation can be categorised as hyperalgesia, that is “an increased amount of pain associated with a mild noxious stimulus or spontaneous pain without any precipitating stimulus” (Cheville *et al.*, 2000; Rang *et al.*, 2007). Some of the known types of chronic pain are: fibromyalgia (a generalised pain of muscles and tissues), back pain, neuropathic pain (arising from damage to the nervous system), arthritis and postoperative pain. Compared to arthritis and back pain, fibromyalgia is the least understood regarding its pathophysiology but is relatively common (2.4% of the population) with a preponderance of cases in women (incidence ratio of 9:1 female:male) (McBeth and Mulvey, 2012). Postoperative pain can be common after surgery to remove a tumour, where the pain stimuli persist after it is healed. In the case of neuropathic pain, it can occur after central nervous system disorders, such as that caused by stroke. Chronic pain is known for its incessant suffering, no matter what is its cause. In the long term it is likely to induce psychiatric disorders like depression that consequently intensifies the complexity of the disease making the treatment even more challenging (Toates, 2007).

1.3.2 The physiology of pain and pathways

Pain is one of the sensory systems that has evolved along with human evolution and was crucial for species survival, in that specific and complex neural pathways developed to transmit a pain signal from sensory cells through the body to the brain and vice versa (Nicholls *et al.*, 2001). Through these signals, it is possible to obtain limb movements (reflex withdrawal) and hence protect the body from harm. The spinal cord represents an important role in contributing to this motor mechanism, since each segment of the

spinal cord contains motor neurones that control a wide range of skeletal muscles that will enable withdrawal response to a painful stimulus (Matthews, 2001).

In the pain pathway the afferent (incoming) signals enter the spinal cord via the dorsal roots and reflex motor pathways exit via ventral roots. In response to the incoming signals the cognitive systems in the brain will activate inhibitory or excitatory transmission through the system (Nicholls *et al.*, 2001). There are several parts of the brain involved in the descending control of pain, some of these structures are the cortex, thalamus, midbrain and periaqueductal grey (Jones and Dunlop, 2007). These brain structures are involved in the process of detecting, suppressing or enhancing the pain sensation. For example, the lateral thalamus has been reported to be involved in the process of discerning the location of injury on the body after acute pain stimuli (Kandel *et al.*, 2000; Squire *et al.*, 2013). Neurons of somatosensory cortex and insular cortex are described as being involved in the aching sensation and overall pain response (Kandel *et al.*, 2000; Squire *et al.*, 2013). Periaqueductal grey interneurons are involved in suppressing pain response by inhibiting the release of neurotransmitters, such as glutamate originating in the primary nociceptive afferents. Injury or abnormality in the parts of the brain involved in the descending pain pathway may also affect perception of pain, as observed in clinical cases of thalamic pain syndrome (Dejerine-Roussy Syndrome) in which mild stimuli are perceived as highly noxious, and cortical abnormality which can cause the condition known as known as “asymbolia for pain” in which subjects lose the unpleasant quality of pain (Kandel *et al.*, 2000; Squire *et al.*, 2013).

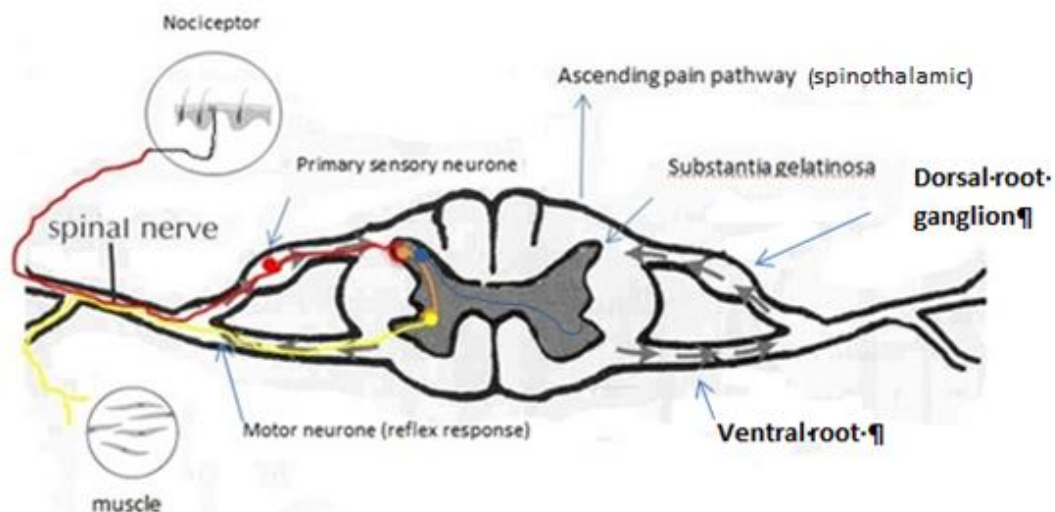


Figure 1 - Pain transmission through the spinal cord

Figure 1 shows details of the incoming signal from a nociceptor in the skin entering the dorsal roots and the reflex motor pathway exiting via ventral roots (figure adapted from <http://www.healingtherapies.info/SCIoverview.htm>).

The transmission from the periaqueductal grey-rostral ventromedial site particularly has the ability to suppress nociceptive inputs conducted through the nociceptors C-fibres and conserving sensory-discriminative information conducted by nociceptor A δ -fibres (Heinricher *et al.*, 2009). This process of descending signal from the brain in response to the painful stimulus involves mainly the neurotransmitters serotonin, noradrenaline and endogenous opioids (J.A.Stamford, 1995).

The different nociceptors, fibre types and neurotransmitters have important functions in the pain pathway and will be explained in the following sections.

1.3.3 Nociceptors

Acute pain and chronic pain are initiated by activation of nociceptors, which are the sensory receptors, that are less sensitive compared to the so-called touch receptors. They are specialised for responding with electrical activity in the event of potential hurt or injury that can be provoked by damaging or penetrating the skin (Matthews, 2001). These sensory receptors are in the periphery of the body and have the function of responding to changes to pressure, temperature and chemicals, and are classified as

mechanoreceptors, thermoreceptors and chemoreceptors, respectively. In general, nociceptors are present in all tissues and have their cell bodies localised in the dorsal root or trigeminal ganglia. These neurones possess a single axon that morphologically bifurcates into a peripheral branch that innervates peripheral target tissue ending in a nociceptor, and a central axon that activates second order neurones that transmit the signal for pain sensation (Woolf and Ma, 2007). The fibres A δ (myelinated) and C (unmyelinated) specially can be activated through pain stimuli caused by intense heat, mechanical stimuli and several chemicals (Caterina *et al.*, 2005). These sensory fibres are located within the skin or in the tissues such as viscera, bone and muscle. Through these fibres the painful stimulus is conducted to the spinal cord; A δ being faster to conduct this signal than the C fibre due to their myelination. Therefore, it is the A δ fibre that provides the sensory stimulus that contributes to generate a withdrawal reflex (Matthews, 2001). After entering the spinal cord the majority of the A δ and C fibres terminate superficially by synapsing with secondary neurones in the grey matter of the dorsal horn (Park *et al.*, 2000a). Most of A δ and C fibres synapse with intermediate neurones in the deeper layers of the dorsal horn; these fibres terminate within spinal cord laminae I-X. The nociceptive information provided by A δ and C fibres are mainly received within laminae I, II, V and VI (Hunt and Bester, 2005). The A δ nociceptors have been subdivided into two main classes named type I and II and classified as mechanical and heat-sensitive nociceptors. The type I fibre has a higher threshold for heat than type II, whereas type II has a higher threshold for mechanical stimuli. Type I can become sensitive to heat after prolonged stimulation, thus it has been suggested that the sensation during first exposure to heat is conducted through the type II (Treede *et al.*, 1998). Some of the transient receptor proteins present in thermal nociceptors and activated by heat or cold have been named as TRPM8, TRPA1, TRPV1, and TRPV2 (Chen *et al.*, 2006a). For the C fibres, they have been classified as polymodal for their ability to respond to thermal, chemical and mechanical stimuli, and these nociceptors are responsible for the slow and long lasting pain sensation after body injury (Taguchi *et al.*, 2010). These slow conducting fibres represent the majority of sensory neurones in the peripheral nervous system (Goodman and Gilman, 2006).

The A δ and C fibres play an important role in the process of pain pathway, which can be explained through the “gate-control pain theory” first proposed by Melzack and Wall (1965). Being a psychologist, Melzack aimed to explain the differences in the level of pain sensation felt by individuals. The principal idea behind this theory is that the

signals received from the nociceptors in the spinal cord, specifically the second order neurones (spinothalamic cells), can be suppressed or enhanced by the inhibitory or excitatory mechanisms controlled by interneurons in the substantia gelatinosa. At this location several chemical events can affect the ascending messages to the brain which are processed in the thalamus, therefore it is known as a “gate for pain transmission” (Melzack and Wall, 1996). This pain pathway is illustrated in Figure 2. This theory revolutionised the concept of pain sensation, and contributed much to explain symptoms such as phantom pain, a type of pain which is provoked by hyperactive second order neurones of the spinal cord caused by damaged or missing C or A δ fibres nociceptors. In the case of phantom limb symptoms, N-methyl-D -aspartate receptors are constantly activated through the damaged C fibre nociceptor and through A δ fibre at the spinal dorsal horn which transmit incessant pain signals through second order nociceptive neurones to the brain (Nikolajsen and Jensen, 2000).

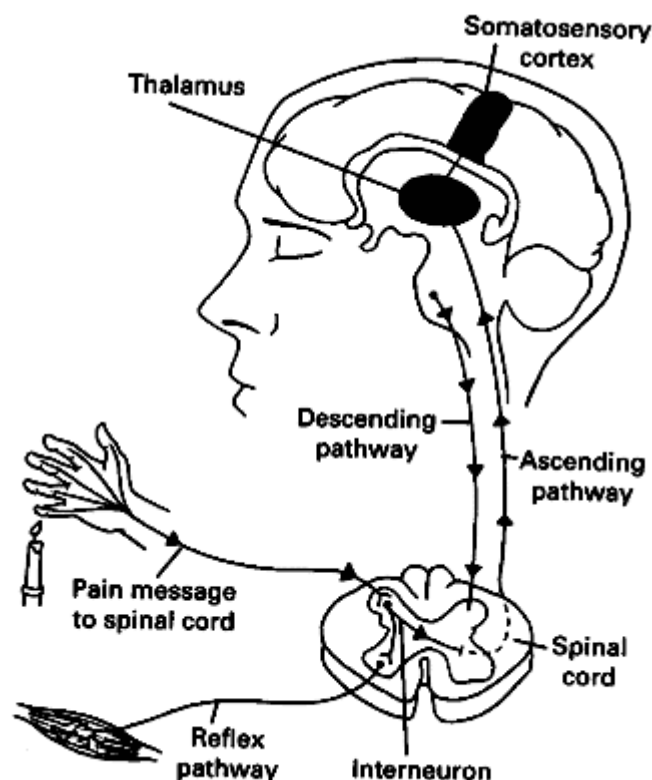


Figure 2 - Basic pain pathway. Figure taken from http://virtual.yosemite.cc.ca.us/dward/physo101/f05_pages/s5_cns/sup_somatosensory_pain.htm.

In the basic pain pathway, shown in Figure 2, the thermal noxious stimuli is transduced in electrical signals that are conducted through nociceptors fibres to the dorsal horn in the spinal cord. This signal can induce withdrawal reflex by synapsing with interneurone to motor neurone. Synapses can also occur between the nociceptors and a second order neurone in the dorsal horn transmit the pain signals through pathways tract such as spinothalamic towards the thalamus and midbrain. In the thalamus synapses will occur with third order neurone that will transmit the pain signals to the somatosensory cortex processing localisation and perception of the pain. The descending pathways transmit signals generated in the brain to the dorsal horn, these signals have the ability to suppress or potentiate the passage of nociceptive messages to the brain.

As described above, for an individual to feel pain sensation caused by an injury, this information is sent from the part of body affected through nociceptors, the information travels through the spinal cord where it can be decreased, increased or blocked before reaching the brain, this is the ascending pathway. Once in the brain, the signal is interpreted as a pain sensation therefore sending a response to withdraw reaction or protection of the area affected, descending pathway (Matthews, 2001). However, the mechanism involved in this process is not as simple as described. The nociceptors conduct this information through action potentials, this electrical signal starts from the location injured that suffered depolarisation caused by the noxious stimulus through chemical mediators such as prostanoids, bradykinin and 5-hydroxytryptamine (Dickenson and Suzuki, 2005). These signals are faster to reach the brain when the nociceptor is myelinated. The depolarisation is caused by changes of cell membrane permeability by special ion channels. For example the increase of sodium ions (Na^+) into the axoplasm in a quantity that reaches above a threshold will trigger action potential (Brown, 2001). The action potential transmission will be propagated by opening and closure of sodium and potassium channels. Some neurones can also be activated through calcium channels. The action potential resulting from these channels are slower and have the ability to produce longer depolarisation (Grahame-Smith and Aronson, 2002). The voltage-gated sodium and calcium channels have been the focus of pain relief drug discovery especially for neuropathic pain, due to the fact that these channels exert an important role in the process of controlling cellular excitability. As a result they have the key for blocking or enhancing pain transmission through action potential. Some sodium blockers are already well known, but because these drugs are not specific they would compromise the body by blocking its normal function

propagation. Therefore, they are most used as local anaesthetic, an example of this type of drug is lidocaine (Zuliani *et al.*, 2010). Consequently, the target of research is to find molecules that potentially can select certain Na⁺ channels subunits that are more specifically involved in pain transmission (Baker and Wood, 2001; Leo *et al.*, 2010).

1.3.4 Neurotransmitter receptors and Pain

There are several neurotransmitters that play an important role transmitting or inhibiting pain, some of them are substance P and glutamate that are usually excitatory. Regarding the inhibitory neurotransmitter, the majority of this synapse is carried out through gamma amino butyric acid and glycine. There are some neurotransmitters that are more involved in the descending pathway transmission process, these neurotransmitters are serotonin, noradrenaline and opiates such as endorphin (Yin *et al.*, 2011; Sacerdote and Levrini, 2012; Wozniak *et al.*, 2012).

Some of these neurotransmitters function at synapses through a diverse range of receptors; therefore some of them have been linked with pain treatment or have been used as the target for drug discoveries. Some of these receptors can be divided into two categories; ligand-gated or ionotropic receptors and G protein-coupled or metabotropic receptors (Iwama and Gojobori, 2002).

The transmission through ligand-gated ion channels is made by opening ionic channels. This process takes place after binding of a neurotransmitter (Novère and Changeux, 1999). Regarding metabotropic receptors, their involvement is far more complex and there are many factors of its mechanism that are still under investigation. However, it has been suggested that the process of depolarisation is carried out for second messengers, as an example phospholipase C (Lee *et al.*, 2004). These receptors are known for being the most abundant and diverse family of cell surface receptors and proteins. These receptors have been reported for being involved in neurotransmission and pain modulation, for this reason they represent a primary target for pain treatment. (Pan *et al.*, 2008). Also they have been suggested to be involved in the process of chronic pathological pain including hyperalgesia, allodynia and paroxysmal or spontaneous pain (Cahill *et al.*, 2007).

Examples of metabotropic receptors are the metabotropic glutamate, muscarinic acetylcholine, GABA_B, norepinephrine, epinephrine, histamine, dopamine,

neuropeptides bradykinin B2, adenosine A1 and endocannabinoids. Examples of ionotropic receptors are 5-HT₃, ATP, nicotinic, TRPV1 for capsaicin and related vanilloids (Lee *et al.*, 2004).

The following sections very briefly outline some of the major receptor classes which have been associated with pain and are, thus, potential targets for antinociceptive agents.

1.3.5 Receptors with potential targets for pain relief

1.3.5.1 Cannabinoid receptor

The cannabinoid receptor also belongs to the G protein-coupled receptor superfamily. These receptors can be activated by the naturally body ligand endocannabinoids and by plant cannabinoids such as tetrahydrocannabinol (Ryberg *et al.*, 2007). The cannabinoid receptors GPCRs, CB1, CB2 and GPR55 are peroxisome-proliferator-activated receptors. These cannabinoid receptors are present in the central and peripheral system and they are involved in the processing of pain signals (Greco *et al.*, 2010). However, its antihyperalgesic and antinociceptive activities result from mediation of several mechanisms that are still under establishment (Akopian *et al.*, 2009).

1.3.5.2 Glutamate and GABA receptors

As mentioned before, glutamate is an important transmitter involved in pain transmission, mainly because it functions at excitatory synapses both at brain and spinal cord levels, its postsynaptic effect operating through ionotropic and metabotropic receptor types. These receptors are classified as alpha-amino-3-hydroxy 5-methyl-4-isoxazepropionic acid (AMPA), N-methyl-D-aspartate (NMDA) and the G-protein-linked metabotropic family of receptors (mGluR5). Research has been focused on these receptors as a target for chronic pain (Kew and Kemp, 2005; Bleakman *et al.*, 2006).

Metabotropic glutamate and metabotropic GABA receptors (specifically GABA_B) are distributed at different levels of the central nervous system, one of their roles involves transmission of nociceptive signals (Goudet *et al.*, 2009). GABA_B receptors are known for controlling neuronal excitability and synaptic neurotransmission modulation. Both receptors have a vital role in many physiological functions and have been associated with several types of neurodegenerative and pathophysiological disorders including chronic pain (Cahill *et al.*, 2007).

1.3.5.3 Lysophosphatidic acid (LPA)

Six G protein-coupled receptors (LPA(1)/Edg2, LPA(2)/Edg4, LPA(3)/Edg7, LPA(4)/GPR23/P2Y9, LPA(5)/GPR92 and LPA(6)/P2Y5) have been identified, that are specific to lysophosphatidic acid (LPA), a phospholipid involved in neuropathic pain that has been identified (Hama and Aoki, 2010). LPA have been reported to activate demyelination of Schwann cells of the periphery and oligodendrocytes linked with the central nervous system. Since it has been suggested that demyelination of fibre is a key factor associated with neuropathic pain, these receptors might represent an important target for chronic pain treatment (Lin *et al.*, 2010).

1.3.5.4 Muscarinic acetylcholine receptors

Muscarinic acetylcholine receptors are G protein-coupled receptors found in the plasma membranes of certain neurones. These receptors exert different roles, including acting as main and end-receptors in the parasympathetic nervous system contributing to the process of releasing acetylcholine (Eglen, 2006). Acetylcholine release in the spinal cord at substantia gelatinosa was observed to induce antinociception by decreasing excitatory transmission induced by glutamate (Jones and Dunlop, 2007).

1.3.5.5 Opioid receptors

The opioid receptors are G protein-coupled receptors, and neurotransmission through this receptor decreases the cyclic adenosine monophosphate involved, that consequently stop depolarisation induced by neurotransmitters such as substance P. There are three protein types that generate opioids peptide, these precursor proteins are proopiomelanocortin, prodynorphin and proenkephalin (Przewlocki and Przewlocka, 2001).

Also there are three types of opioid receptors that are linked with antinociception. These receptors are described as μ , δ and κ types (Kosterlitz and Paterson, 1985). The μ receptor is most known as the morphine receptor type, perhaps one of the most important studies leading to its establishment was carried out by Martin (1979). Opioid receptors and its target for drugs will be discussed later in this chapter.

1.3.5.6 Serotonin receptors

Most of the serotonergic receptors are G protein-coupled receptors, only the 5-HT₃ is a ligand-gated ion channel (Hannon and Hoyer, 2008). The activation of presynaptic 5-HT₃ by 5-HT receptors on central terminals of spinal afferents, induces the increase of transmission in the spinal cord via the dorsal horn and provokes a pain increase and reflex responses. Inhibition of 5-HT₃ receptors has been shown to prevent the development of chronic pain in rats (Suzuki *et al.*, 2004).

1.3.5.7 Somatostatin and galanin receptors

The neuropeptide somatostatin, also known as somatotropin, has been reported for its involvement during painful stimuli as an inhibitory activator. It seems that this activity is mediated by five G protein-coupled receptors. Research has shown that when these receptors are activated it reduces pain sensation (von Banchet, 2008). Galanin known as GalR1, GalR2 and GalR3 are neuropeptide with G-protein-coupled receptors that assist with synapse function in the central, peripheral and in the endocrine system. These receptors have also been targeted for the treatment of pain (Mitsukawa *et al.*, 2008).

As described these receptors represent an important target for pain relief drugs thus giving perspective and hope for sufferers that in the near future many of their symptoms could be treated. However, pain receptors are far from being the only target of research nowadays, another line of research has been the focus to understand the difference of pain threshold between individuals based in their genes and gender, the idea behind these studies is that it might be more effective to treat pain based in each individual variability condition. The next section briefly describes some of these results.

1.3.6 Gender and genetic differences involved in pain perception and modulation

Studies have been discussing the implication of gender differences in pain perception. Although it is a controversial area it has shown some very interesting facts. The majority of studies hypothesising gender differences are carried out with individuals suffering from irritable bowel syndrome (IBS), due to the prevalence of this symptom in woman. Research has shown that women have a lower threshold to rectosigmoid distension than men (Chang *et al.*, 2006). Also, reports have suggested that several areas of male and female brains can react differently when given the exactly same pain stimulus provoked by IBS. The female brain demonstrated to have higher activation in

the ventromedial prefrontal cortex, right anterior cingulate cortex, and left amygdala, which are involved as “emotion based centres”, however in the male brain this activation was reported to be predominant in the right dorsolateral prefrontal cortex, insula and dorsal pons/periaqueductal gray regions (Naliboff *et al.*, 2003). Some of these variations could be the result of genetic differences related to the sex chromosomes where the hormones testosterone and estradiol influence sensitivity to pain as well as its modulation, therefore the difference of concentration of these hormones would influence pain perception according to the gender (Foulkes and Wood, 2008).

Although the pain physiological pathway and its molecular mechanism present similarity between species and individuals, there are established differences in pain modulation and perception caused and influenced by genetic variability and environment. These variations of mutation in the genes can increase the propensity to feel pain or lose it completely by having total insensitivity to painful stimuli. An example of pain insensitivity caused by mutation was reported by a study involving consanguineous families, where the mutation in the gene SCN9A encoding the alpha subunit of Na v1.7 voltage-gated sodium channel can cause complete inability to sense pain (Cox *et al.*, 2006). Further studies involving gene influence in pain perception have shown that single nucleotide polymorphisms have a substantial impact on pain sensitivity. These nucleotide polymorphisms were observed in the genes for catechol-O-methyltransferase that facilitates the inactivation of catecholamine neurotransmitters, for the melanocortin-1-receptor, also for the μ -opioid receptor OPRM1 (A80G, A118G), and for tetrahydrobiopterin (BH4) that is an important cofactor in nitric oxide, serotonin, and catecholamine production. These genes are all involved in pain sensitivity variations and also contribute to the mechanism of pain inhibition undertaken by endorphins, μ -opioids and κ -opioids (Foulkes and Wood, 2008).

So far this chapter has described pain propagation and some of the mechanism involved, the aim of next section is to present some of the pain relief treatment and also its action mechanism.

1.3.7 Pain management and mechanisms of pain relief

The relief of pain has been a major target of pharmacotherapy and drug discovery for centuries. Several plants such as *Papaver somniferum* and *Salix* family have been used

to relieve pain for thousands of years. Their active agents have been the precursors of pain relief drugs and their compounds have elucidated and established the opiates and cyclooxygenase inhibition in the mechanism of pain moderation. These drugs have enabled medicine to have better control and manipulation of pain (Brune, 2002). However, these drugs are not without their side effects and several pain symptoms are still untreatable, therefore the pharmaceutical industries are in a competitive search for new or more efficient drugs, especially for symptoms like chronic pain, where many treatments have failed (Jensen and Finnerup, 2007).

Drugs for the treatment and management of pain (analgesics), can be broadly placed into two categories: opioids and non-opioids (Park *et al.*, 2000b).

1.3.7.1 Opioids

Opioid compounds, such as morphine from *Papaver*, have been used for thousands of years and historically represent one of the oldest treatments used for pain relief. The activities of opioids, such as heroin and morphine, occur as a result of “mimicking” the body’s naturally occurring substances. These substances are usually endogenous opioid peptides which occur as three families: endorphins, enkephalins and dynorphins. These peptides are present in the spinal cord, brain and peripheral nerves (Grahame-Smith and Aronson, 2002). The production of these opioid peptides by the body can be triggered by immune cells such as T and B-lymphocytes, monocytes and macrophages in response to injury, as well as in response to painful stimuli. The general mechanism of action induced by opioids is described acting as central rather than peripheral, despite opioid receptors also being located on the peripheral terminals (Kandel *et al.*, 2000). The opioid activation reduces pain transmitter release by opening potassium or closing calcium channels (Shipton, 1999). The sites of action of opioids have been reported to be localised in the spinal cord (substantia gelatinosa), midbrain and thalamus (Chahl, 1996; Kumamoto *et al.*, 2011).

As described in the receptor section, opiate receptors are classified as three subtypes, μ , δ and κ . The analgesic drugs that have their effects through opiate receptors act as partial or total agonists. Some agonists of subtype κ act by closing calcium channels and activation of these receptors has been shown to have an effect that is rather more dysphoric than analgesic. Therefore the theory is that δ -opioid receptors might have a much lower drug dependence than μ -opioid (Waldhoer *et al.*, 2004). Most opioid drugs

act at the μ receptor and opiates acting at δ receptor are less effective for visceral pain. There is a high concentration of μ receptors in the periaqueductal gray matter which is substantially involved in suppressing pain through the descending pathway regulation (Kandel *et al.*, 2000). The μ and δ receptor operates by opening of potassium channels, the drug morphine acts at δ and κ receptor, therefore it is a drug that induces analgesia and dysphoria, also in some cases it causes dependence (Shipton, 1999; Carr and Mague, 2008). Morphine is mainly used to treat severe pain and despite its high indices of addiction it remains the most efficient drug available to treat cancer pain (Bonica *et al.*, 2009). Some researchers have been involved in projects for morphine molecule modification and have delivered versions known as diamorphine or heroin and hydromorphone. However, in the course of time, heroin presented to be more highly addictive than morphine. Further investigations have found a possible less addictive opium molecule named as codeine. But it is more recommended as a mild pain reliever (Grahame-Smith and Aronson, 2002).

Although morphine was one of the first opioids in the market, its mechanism of action still need clarifications. Morphine efficiently activates G protein signals but inefficiently induces endocytosis, this fact has been associated with the receptor desensitisation and drug tolerance (Dang and Christie, 2012). Due to several sides effects caused by morphine as well some inefficiency as an anesthetic, an alternative for this drug was investigated, and in 1960 a more powerful synthetic opioid named as fentanyl was announced (Stanley, 1992). Fentanyl is known for acting quickly for a short period. For example, intranasal fentanyl has a half-life of 6.5 minutes. Although, it is more powerful than morphine it is only more recommended for acute severe pain than chronic pain (Foster *et al.*, 2008).

As mentioned, δ -selective agonists are a promising target for drug development, particularly for chronic pain where morphine, despite its dependence problem, is still the recommended drug. Preclinical studies have presented positive results for δ -selective agonists where they demonstrated to have antinociception in several chronic pain models including neuropathic and cancer pains. However so far, there are not δ opioid drugs available. The drug Snc-80 (GSK, NIH) is one of the leading δ opioids but it is still under development (Walwyn *et al.*, 2010; Nagase and Fujii, 2011).

1.3.7.2 Non-opioids

Non-opioid analgesic drugs can be generally represented by aspirin, related salicylates and paracetamol. Aspirin and other salicylates belong to the group known as the non-steroidal anti-inflammatory drugs or NSAIDs. The NSAIDs are usually available as over the counter drugs and are among the most used drugs worldwide (Ray *et al.*, 2001; Grahame-Smith and Aronson, 2002).

Regarding their mechanism of action, they block the production of certain prostanoids such as prostaglandin. This blockage is obtained through inhibition of the enzyme responsible for their production, the enzyme cyclo-oxygenase (COX). This enzyme synthesises prostaglandin from arachidonic acids (Shipton, 1999).

In the process of pain, prostaglandin acts as a lowering pain threshold inducer, it facilitates the neurone to fire and deliver pain signals. The inhibition of prostaglandin by NSAIDs increases the body's threshold for pain, making the neurones less sensitive to pain stimuli (Grahame-Smith and Aronson, 2002). When NSAIDs mechanism was first unveiled, it was suggested that their activities only involved a peripheral mechanism, however more recently studies have supported that they might also have central involvement in their actions (Samad *et al.*, 2002).

Further studies have found that there are isoforms of the COX enzyme, which are known as COX-1 and COX-2. The enzyme COX-1 appears to be more involved with normal physiological function occurring in the body. For example, it is involved in the gastrointestinal protection process. Therefore, analgesic inhibiting COX-1 such as aspirin have been causing gastrointestinal irritation and bleeding. The COX-2 enzyme is more involved in the process of inducing hypoxia, and inflammatory stimuli (Shipton, 1999). Hence, it attracted the attention of the drug industry to search for selective COX-2. These drugs came to be marked as celecoxib which is a sulfonamide-containing 1,5-diarylpyrazole and some of them have the brand name celebrex. However, later on researchers found out that although it might be safer in terms of gastrointestinal bleeding it was increasing the risk of cardiovascular dysfunction. The reason for these events is due to the fact that COX-2 inhibitor only decreases prostacyclin and not thromboxane. The availability of this prostanoid in the system without its functional competitor results in an increase of blood vessel constriction and platelet-aggregation,

thus leading to thrombosis and heart failure (Kearney, 2006; Funk and FitzGerald, 2007).

On the other hand, drugs such as paracetamol or acetaminophen, despite the fact that their mechanism still needs deep clarification, are a weak COX inhibitor for which COX selection occurs in the brain. For this reason they have been used as an alternative analgesic by people with haemophilia and heart disease (Rang *et al.*, 2007).

1.4 The Genus *Hyptis* Jacq: Chemical and Biological Studies

1.4.1 Hyptis genus distribution and the Hyptis crenata species

Plants from *Hyptis* genus are fast growing and perennial. In general *Hyptis* species contain essential oil which generates interest about its volatiles properties (Noudjou *et al.*, 2007b; Tonzibo *et al.*, 2009; Zellner *et al.*, 2009). There are over 300 species of *Hyptis* that are mainly found in the tropical Americas and African savannah areas (Harley, 1988; Ogunbinu *et al.*, 2009). There are several of these species that have been reported in the Brazilian territory, specifically found in the Cerrado that is also a tropical savannah area (Zellner *et al.*, 2009). The species *Hyptis crenata* Pohl ex Benth studied in this PhD project mainly grows in the central west of Brazil (Pott, 1997). A photo of *H. crenata* growing in Mato Grosso state is displayed in Figure 3. *H. crenata* is characterized by being a subshrub from 0.3 to 0.8 m height with a strong smell of mint. It has flowers and leaves almost all year round except for August-September when the aerial part dies. It is one of the preferable plants used by the honeybee, and due to the eugenol compounds usually present in the plant, the honey produced has a clove scented flavour (Pott, 1997).

Figure 4 shows the distribution of where the *Hyptis* genus grows throughout the world. The *Hyptis* growing in Australia is an alien invasive from tropical America, this species is *H. suaveolens* and has been considered as a problematic weed for Australian agriculture (Cowie and Werner, 1993; Kissinger, 2003).



Figure 3 - Photograph of *Hyptis crenata* growing in Mato Grosso, Brazil

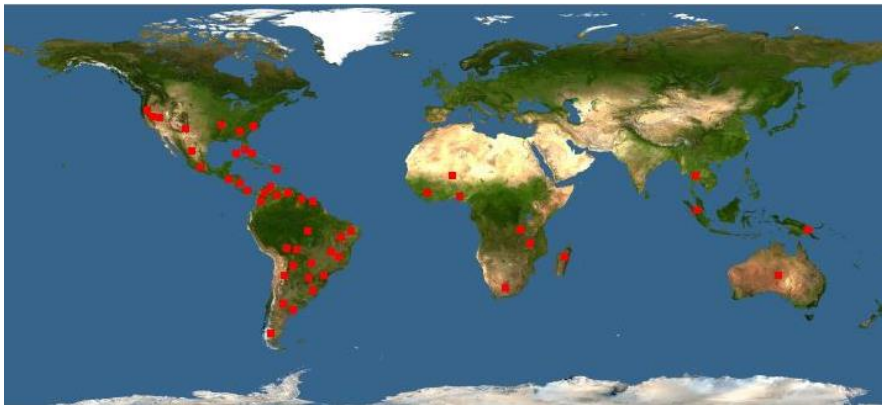


Figure 4 - *Hyptis* genus world distribution.

Source: <http://www.discoverlife.org/hyptis>; year: 2009

1.4.2 Traditional use of *Hyptis* species

Species from the genus *Hyptis* Jacq have been used widely in traditional medicine mainly in South America and Africa for treatment of various illnesses, such as rhinopharyngitis, nasal congestion, skin diseases, gastric disorders, fever and pain. Some *Hyptis* species are used as anticancer and antifertility agents (Menezes *et al.*, 2007; Tonzibo *et al.*, 2009). Some *Hyptis* species are especially used for fungal infections or other dermatitis (Celine *et al.*, 2009). The tea from leaves have been reported to be used as a diaphoretic, tonic, stimulant to treat inflammation of eyes and throat, constipation and arthritis (Van de Berg, 1993).

The only available traditional use survey regarding *H. crenata* as medicinal use was reported by De Jesus *et al.* (2009). This study was carried out in Distrito de Pirizal, Mato Grosso, Brazil. Thirty eight people were interviewed. Among the 49 species mentioned, *H. crenata* Pohl and *H. suaveolens* were the most indicated to antiulcer and anti-inflammatory effects. The study showed that they used *Hyptis* species leaves or the entire plant as a tea or bath. *Hyptis* leaves were also reported to be used as insect repellent when rubbed on the skin (Pott, 1997) and *Hyptis* flowers have been used as a source of nectar for production of honey and propolis, known to contain antibacterial compounds (Park *et al.*, 2002; Santos *et al.*, 2003).

1.4.3 Biological studies reported for *Hyptis* genus

Although the traditional use of *H. crenata* has been reported, as mentioned above, there have been no reports about pharmacological studies of this species. On the other hand, there are a number of reports of biological activities of other *Hyptis* species, as presented in Table 1.

Activities	Species	Authors
Insecticidal	<i>H. fruticosa</i> , <i>H. martiussi</i> , <i>H. pectinata</i> , <i>H. suaveolens</i> , <i>H. spicigera</i> <i>H. verticillata</i>	(Buchanan <i>et al.</i> , 2000; Araujo <i>et al.</i> , 2003; Boeke <i>et al.</i> , 2004; Amusan <i>et al.</i> , 2005; Facey <i>et al.</i> , 2005; Kouninki <i>et al.</i> , 2005a; Kouninki <i>et al.</i> , 2005b; Omolo <i>et al.</i> , 2005; Jaenson <i>et al.</i> , 2006; Sanon <i>et al.</i> , 2006a; Sanon <i>et al.</i> , 2006b; Ngamo <i>et al.</i> , 2007; Ngassoum <i>et al.</i> , 2007; Njan Nlôga <i>et al.</i> , 2007; Noudjou <i>et al.</i> , 2007b; Noudjou <i>et al.</i> , 2007a; Oliva <i>et al.</i> , 2007; Silva <i>et al.</i> , 2008; Bum <i>et al.</i> , 2009; Ladan <i>et al.</i> , 2009; Misra, 2009; Othira <i>et al.</i> , 2009; Conti <i>et al.</i> , 2010; Ilboudo <i>et al.</i> , 2010; Ladan <i>et al.</i> , 2010; Conti <i>et al.</i> , 2011)
Acaricidal	<i>H. verticillata</i>	(Porter <i>et al.</i> , 2009)
Antibacterial and antifungal	<i>H. brevipes</i> , <i>H. crenata</i> , <i>H. fasciculata</i> , <i>H. martiusii</i> , <i>H. mutabilis</i> , <i>H. ovalifolia</i> , <i>H. pectinata</i> , <i>H. sidifolia</i> , <i>H. spicigera</i> , <i>H. suaveolens</i> , <i>H. verticillata</i>	(Goun <i>et al.</i> , 2003; Souza <i>et al.</i> , 2003; Stadnik <i>et al.</i> , 2003; Fragoso-Serrano <i>et al.</i> , 2005; Krishnamurthy and Shashikala, 2006; Mahida and Mohan, 2006; Oliva <i>et al.</i> , 2006; Nascimento <i>et al.</i> , 2008; Sharma and Tripathi, 2008; Coutinho <i>et al.</i> , 2009; Ladan <i>et al.</i> , 2009; Busmann <i>et al.</i> , 2010; Coutinho <i>et al.</i> , 2010; Mbatchou <i>et al.</i> , 2010)
Anti-HIV activities	<i>H. capitata</i> , <i>H. lantanifolia</i> , <i>H. atrorubens</i>	(Kashiwada <i>et al.</i> , 1998; Antoun <i>et al.</i> , 1999; Matsuse <i>et al.</i> , 1999)
Antiplasmodial, antitrypanocidal and leishmanicidal activities	<i>H. suaveolens</i> , <i>H. brevipes</i> , <i>H. stellulata</i> , <i>H. lacustris</i>	(Ziegler <i>et al.</i> , 2002; Bourdy <i>et al.</i> , 2004; Abe <i>et al.</i> , 2005; Chukwujekwu <i>et al.</i> , 2005; Celine <i>et al.</i> , 2009)
Antioxidant, dermatological and anti-aging activities	<i>H. fasciculata</i> , <i>H. martiusii</i> , <i>H. suaveolens</i> , <i>H. crenata</i> , <i>H. fasciculata</i> , <i>H. heterodon</i>	(Shirwaikar <i>et al.</i> , 2003; Silva <i>et al.</i> , 2005; Kolawole <i>et al.</i> , 2009; Silva <i>et al.</i> , 2009; Simionatto <i>et al.</i> , 2010)
Anti-cancer and proliferative activities	<i>H. brevipes</i> , <i>H. capitata</i> , <i>H. emoryi</i> , <i>H. fasciculata</i> , <i>H. martiusii</i> , <i>H. tomentosa</i> , <i>H. suaveolens</i> <i>H. verticillata</i>	(Sheth <i>et al.</i> , 1972; Yamagishi <i>et al.</i> , 1987; Lee <i>et al.</i> , 1988a; Lee <i>et al.</i> , 1988b; Yamagishi <i>et al.</i> , 1988a; Yamagishi <i>et al.</i> , 1988b; Kashiwada <i>et al.</i> , 1998)

Activities	Species	Authors
Anti-cardiovascular	<i>H. fruticosa</i>	(Santos <i>et al.</i> , 2007a)
Anti-inflammatory	<i>H. brevipes</i> , <i>H. suaveolens</i> , <i>H. verticillata</i> , <i>H. martiusii</i>	(Kuhnt <i>et al.</i> , 1994; Gonzalez <i>et al.</i> , 1995; Shenoy and Shirwaikar, 2002; Grassi <i>et al.</i> , 2006; Raederstorff <i>et al.</i> , 2008)
Anti-depressive	<i>H. pectinata</i>	(Bueno <i>et al.</i> , 2006)
Antinociceptive	<i>H. fruticosa</i> , <i>H. pectinata</i> , <i>H. suaveolens</i>	(Bispo <i>et al.</i> , 2001; Lisboa <i>et al.</i> , 2006; Marcal <i>et al.</i> , 2006; Menezes <i>et al.</i> , 2007; Santos <i>et al.</i> , 2007b; Arrigoni-Blank <i>et al.</i> , 2008; Raymundo <i>et al.</i> , 2011)
Anti-ulcer	<i>H. mutabilis</i> , <i>H. crenata</i> , <i>H. spicigera</i>	(Barbosa and Ramos, 1992; De Jesus <i>et al.</i> , 2009; Takayama <i>et al.</i> , 2011)

Table 1 - Biological activities reported for species of the genus *Hyptis*

The majority of *Hyptis* experimental studies involved testing the essential oil and these species were reported to have activities such as insecticidal, fungicidal, anti-HIV and antiplasmodial, although there are *Hyptis* species for which these effects were not confirmed by these experiments (Almtorp *et al.*, 1991; Antoun *et al.*, 1999; Bourdy *et al.*, 2004). This factor could be due to genotype and the environmental condition that plays a very important role in the type and quantity of compounds found in the plant essential oil (Pino *et al.*, 2002; Martins *et al.*, 2006; Koba *et al.*, 2007; Ngassoum *et al.*, 2007; Sales *et al.*, 2007; Silva *et al.*, 2007; Grassi *et al.*, 2008).

The anti-inflammatory effects of *Hyptis* also have been the subject of study of several researchers. The compound sideritoflavone from *H. verticillata* was suggested for being responsible for its anti-inflammatory effect by inhibiting prostaglandin release (Kuhnt *et al.*, 1994). Also, suaveolol and methyl suaveolate, both diterpenes from *H. suaveolens* are mentioned with this effect. According to the research these compounds showed nearly the same dose-dependent topical anti-inflammatory activity, only two to three times lower than indomethacin (Grassi *et al.*, 2006). In addition, *H. brevipes* ethanolic extract has been reported with a high capacity of xantine oxidase inhibition and also suggested as an alternative treatment for gout that is considered as an acute case of inflammation (Goun *et al.*, 2003). Two patents of compound carnosic acid 12-methyl ether found in *Hyptis* have been registered for the treatment or prevention of inflammatory disorders involving joints (Raederstorff *et al.*, 2008).

As mentioned in the previous section, *Hyptis* genus has always been linked with stomach discomfort relief in traditional use medicine. Possibly due to this association this species has been tested for antiulceration effects (Barbosa and Ramos, 1992; Takayama *et al.*, 2011). This research showed that the essential oil of *H. mutabilis* was able to reduce ulcers induced by indomethacin, a non-steroidal anti-inflammatory drug that works by inhibiting the production of prostaglandins (Flower, 1974). The interesting fact from this result is that the methanolic extract of *H. mutabilis* has also been reported for anti-inflammatory properties (Melo, 2003). Although the mechanism of this effect is not described, it might be different from the anti-inflammatory effect of *H. verticillata* whose compounds decrease prostaglandin levels (Kuhnt *et al.*, 1994) because prostaglandin inhibitors drugs are known for causing gastric damage (Takeuchi *et al.*, 2005), this is not the case of *H. mutabilis*, that has the ability to inhibit the ulcer formation induced by indomethacin. Therefore, from these studies it could be suggested that although some different species of *Hyptis* can have the same activities, it is possible that this effect can be obtained through different mechanisms induced by different compounds. Alternatively it could be that the *H. mutabilis* treatment tested in the anti-inflammatory experiment had compounds such as sideritoflavone, and that this type of compound was not present in the anti-ulcer treatment due to the extraction methods and/or environmental condition where the plant was growing.

The antidepressive effect of *H. pectinata* leaves was tested in mice and rats by Bueno *et al.* (2006). The authors reported that a significant effect of the aqueous extract was observed in the open field, forced swimming and apomorphine-induced hypothermia, but no effects on elevated plus maze and sleep test. Although according to this article *H. pectinata* compounds might be involved in the anxiolytic linked anti-depressant effects, several publications, as will be described later in this section, also reported antinociceptive activity of *H. pectinata*. This antinociception effect can be playing a role in enhancing the threshold of hypothermia and swimming since pain can be involved in both procedures (Mahesh *et al.*, 2010), therefore it could be a false positive result for antidepressant effect.

There are four studies that reported the antinociceptive activity of *H. pectinata*, these studies evaluated this property using several extraction methods; aqueous, hexane, chloroform, ethyl acetate and essential oil. In the hexane, chloroform and ethyl acetate extracts the researchers suggested an opioid-like effect and supraspinal site of action

(Bispo *et al.*, 2001; Lisboa *et al.*, 2006; Arrigoni-Blank *et al.*, 2008; Raymundo *et al.*, 2011), while aqueous extraction was indicated as acting in the cyclooxygenase and lipoxygenase pathways (Bispo *et al.*, 2001). Different genotypes of *H. pectinata* were evaluated by Arrigoni-Blank *et al.* (2008). They described that the essential oil from these genotypes showed both peripheral and central effects and suggested that these effects were due to the interaction of all constituents present in the essential oil samples, not only the majority compounds, in this case indicated as β -caryophyllene and pectinone. The species *H. suaveolens* and *H. fruticosa* were also tested for antinociception using aqueous extract, ethyl acetate extract and the essential oil (Marcal *et al.*, 2006; Menezes *et al.*, 2007; Santos *et al.*, 2007b). For the *H. suaveolens* study involvement of the opioid system in a central site of action was suggested, while the latter study suggested peripheral activity. The authors have suggested that sesquiterpenes and monoterpenes could be playing an important role in these antinociceptive effects. The majority of these antinociceptive studies have indicated that the non-polar compounds of *Hyptis* tested are likely to be acting as opioid effect whereas the more polar compounds have their activities through cyclooxygenase and lipoxygenase mechanism. However it is still not clear which specific compound(s) is (are) responsible for these effects.

The doses applied in these studies were mainly 100, 200 and 400 mg/kg, without justification for the amounts used or explanation if they were based in traditional use research. The majority of these studies reported that the *Hyptis* treatments were not dose-dependent (Bispo *et al.*, 2001; Lisboa *et al.*, 2006; Marcal *et al.*, 2006; Menezes *et al.*, 2007; Santos *et al.*, 2007b; Arrigoni-Blank *et al.*, 2008; Raymundo *et al.*, 2011). Perhaps this lack of dose dependence response is due to the doses given to the animals. Because the range of these doses was from 100 to 400 mg/kg, therefore they might be too high in relation to the receptor occupancy that reached the maximum response (Adler and Geller, 1984; Lutz *et al.*, 2006).

1.4.4 Overview of chemical compounds reported from the genus *Hyptis*

The chemistry information of *H. crenata* species have been reported in three studies however all of them are describing only volatile compounds. These compounds were terpinolene, β -caryophyllene, 1, 8-cineole, borneol, β -caryophyllene, α -pinene, limonene, β -pinene, camphor (Scramin *et al.*, 2000; Pino *et al.*, 2002; Rebelo *et al.*, 2009). The compounds α -pinene, 1,8-cineole, β -pinene, camphor, limonene and γ -

terpinene were part of the composition of an *H. crenata* preparation that was effective as an antioxidant (Rebelo *et al.*, 2009).

For the genus *Hyptis* in general, the diterpenes were compounds frequently associated with reports of insecticidal, anti-inflammatory, antiplasmodial and cytotoxic effects. Also, sesquiterpenes and monoterpenes are reported as antinociceptive and anti-inflammatory.

Some of the chemical compounds described for *Hyptis* genus are listed in Appendix A. The table includes the compound name, molecular formula, molecular weight, the *Hyptis* species that contained the compound and pharmacological effect(s) ascribed to this compound in the literature.

In the literature the 5 compounds described in the highest number of papers reporting chemical constituents of *Hyptis* plants were: β -carophyllene (in 29 papers), Trans- α -bergamotene (15), Eucalyptol (1,8 cineole) (14), Sabinene (14) and Germacrene D (9).

In addition, the topic about compounds from the *Hyptis* genus will be further explored in the chemical analyses of *Hyptis crenata* decoction and its fractions, in Chapter 7.

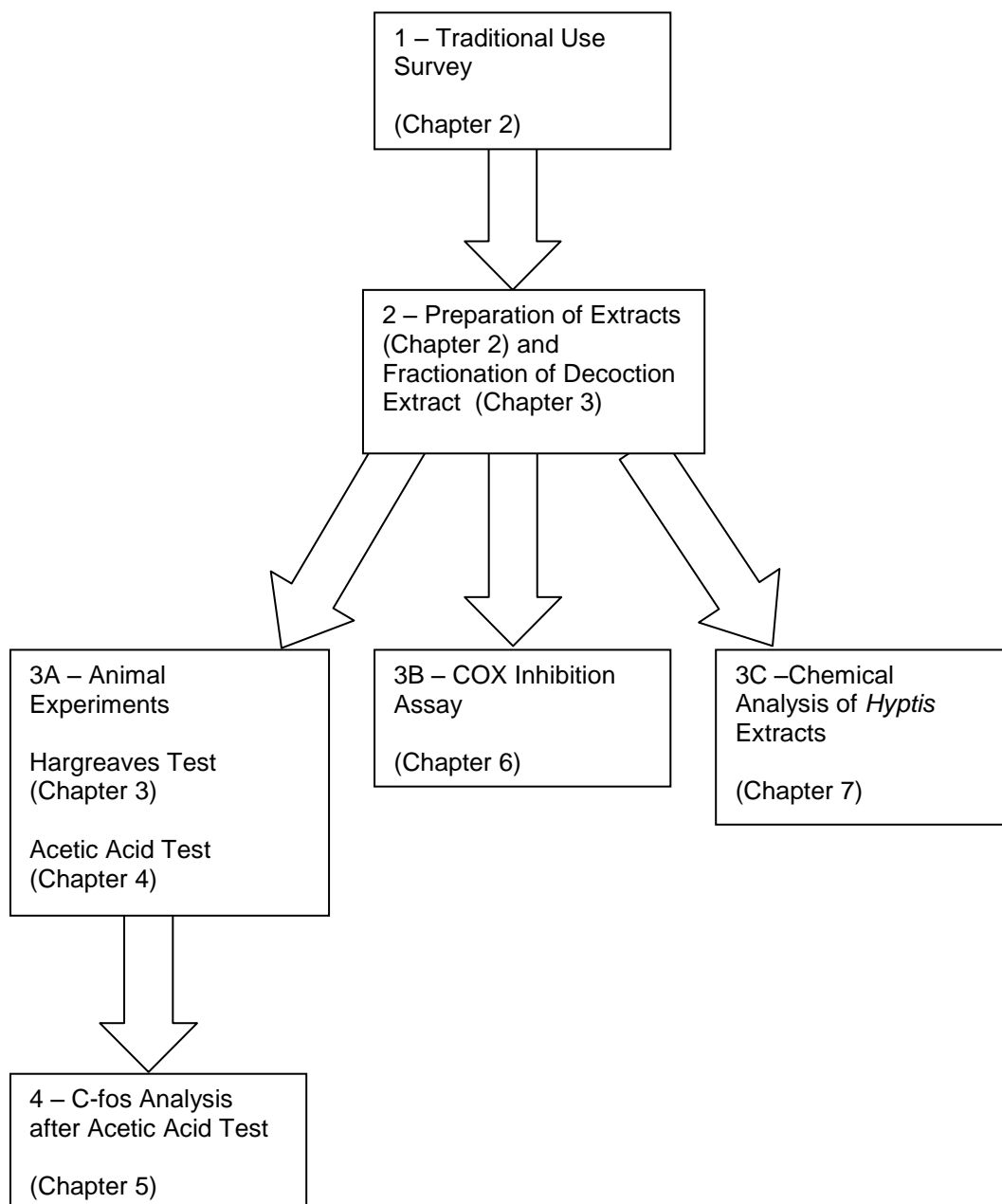
1.5 Aims

The overall objective of this project is to evaluate the antinociceptive properties of *Hyptis crenata* Pohl. The specific aims are:

1. To determine the traditional methods for preparing *H.crenata* and its uses;
2. Investigate the antinociceptive effects of *H. crenata* Pohl using the extraction mentioned in the traditional use in animal models;
3. Investigate the *H. crenata* chemical compounds and mechanisms involved in the antinociceptive activity.

1.6 Overall Plan for the Study

1.6.1 Time-line diagram

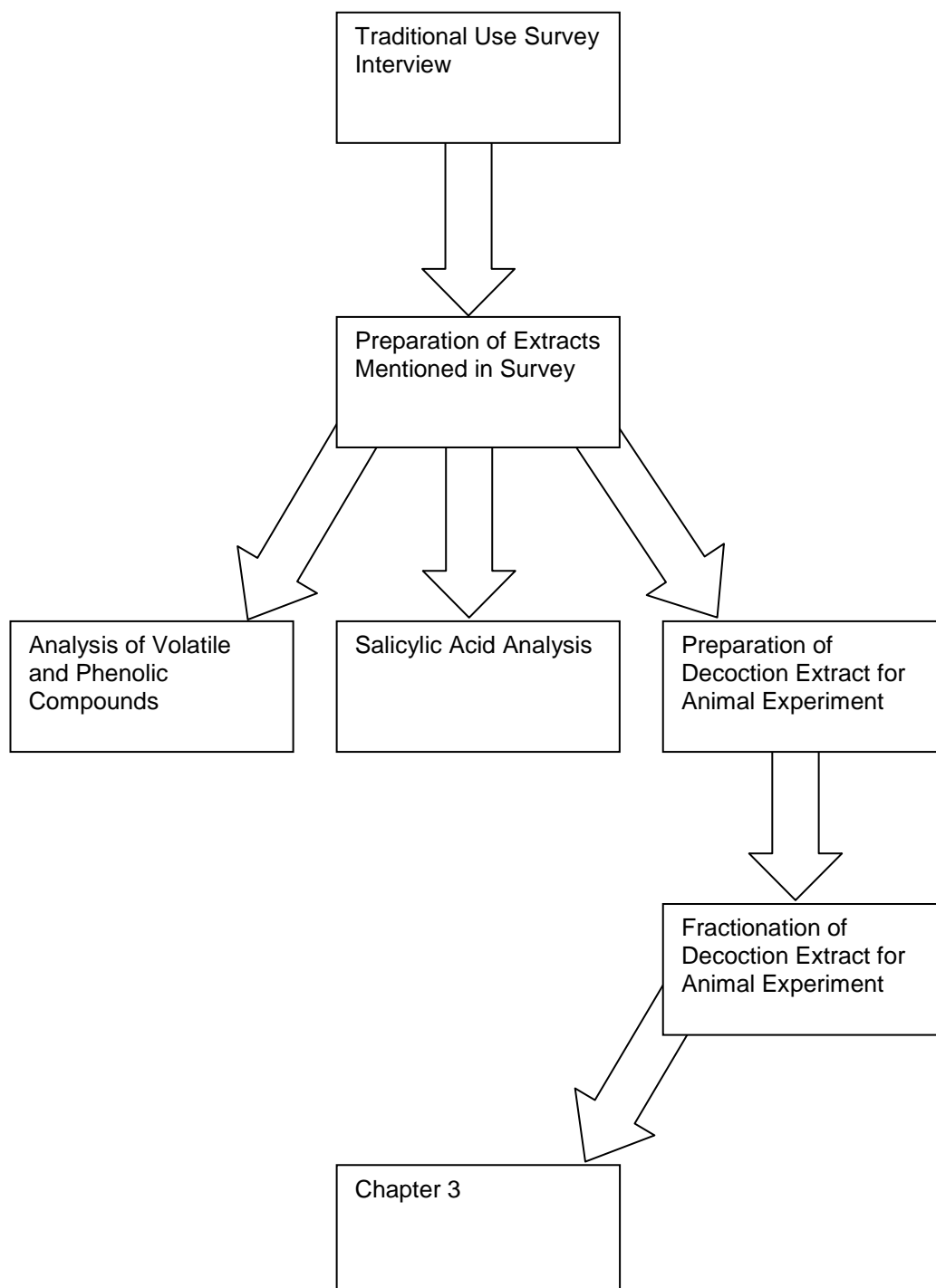


Overall the goal of this thesis was to investigate the antinociceptive activity of *H. crenata* (reported in the traditional use survey) and its mechanism. Therefore, *H. crenata* decoction extract and its fractions were tested through animal trials, brain analysis was performed for the animals treated with the decoction extract to measure Fos protein expression and also the decoction extract and its fractions were evaluated for their potential as COX inhibitors. In addition, chemical analysis of these treatments was performed in order to identify which compound(s) might be playing an important role in this effect.

Chapter 2. Traditional Use of *Hyptis crenata* Pohl

2.1 Overall Procedures

2.1.1 Flowchart



2.2 Traditional Use and Method of Preparation of *Hyptis crenata* Pohl

As described in the *Hyptis* genus studies (Section 1.4), *Hyptis crenata* species have been mainly referred to in papers on traditional medicine for treatment of gastric disorders and pain related symptoms. However, there were no available details of its preparations or its specific use. Therefore, the objective of this survey was to investigate and describe the traditional methods of preparation and utilisation of *Hyptis crenata* Pohl, as background information for an evaluation of the described effects and the plant preparation through pre-clinical trials.

2.2.1 Material and methods

2.2.1.1 Recruitment of interviewees

The first step was to interview users of *H. crenata* to find out what *H. crenata* was used for, and its methods of preparation. The aim was to investigate at least 20 users located in the Vila Bela and Porto Esperidiao regions. The procedure to approach the users was; showing the plant at strategic places such as supermarkets or direct visiting homes, enquiring if they knew the plant and if they use it. Figure 5 shows an example of this approach with one of the people interviewed. The criterion for participation was that a person had used *H. crenata* and knew how it had been prepared.



Figure 5 - *Hyptis crenata* being shown as a method of approach to check if a person knew and used it

2.2.1.2 Method of collecting data

The interviews were conducted using questionnaires conducted with 20 people, with open and closed questions. With the permission of the interviewee the conversation was recorded, later the recorded interview was listened to and any missing information in the written questionnaire was added.

The questionnaire is presented in Appendix B of this thesis. The main questions were: What was *H. crenata* used for, how was it prepared and how much was consumed?

The reason for asking these and other questions written in the questionnaire was to be able to reproduce a *H. crenata* treatment to be tested in the pre-clinical trial that would be similar to what people have been using. This should include for example the type and concentration of plant material in the preparation, volume of intake, administration route, etc. In addition, these results would contribute to the choice of animal model and

other test to be used. Similar questionnaires have been applied in ethnobotanical studies, although these questions were usually less specific about the details of method of plant preparation (Rodrigues and Carlini, 2006; Souza, 2007).

The amount of plant material (e.g. number of leaves, branch etc.) mentioned for making the preparation was weighed in order to calculate the amount in grams.

2.2.2 Results

The interviews were carried out with 10 females and 10 males, with a median age of 48 years. None of the individuals were from the same household, so the same data were not repeated.

The survey results showed that *H. crenata* has been used mainly for pain related purposes; it was used to relieve forms of mild pain, headache, stomach discomfort, menstrual pain and inflammation as shown in Table 2. It was also mentioned by two of the interviewees that they knew of *H. crenata* being used by others to treat prostate cancer, although they had not used it themselves for this purpose.

When the plant was used to treat headache and stomach discomfort the administration route was orally through drinking the preparation. For treating flu, fever and infections they used *H. crenata* medication as a bath.

When using the tea to treat worms, the people described drinking the plant preparation once per day for 2 to 3 days. Interviewees reported using the treatment for just 1 day to treat headaches and fever, 2 to 3 days to treat uterus and bladder infection and 6 days for using *H. crenata* for “blood cleansing”.

The interviewees answered that they were aware that it is not used to cure illness, it is only used to relieve the symptoms. They also reported that it was not taken as a regular drink, only as a medication (15/20). Some of the people had been using the plant since childhood (8/20). The knowledge of using the plant had usually been taught by a parent, grandparent or parent-in-law (14/20). The majority of interviewees indicated that this medication can be taken any time of the day (19/20), also the plant material can be collected at any time of the day (16/20) and the year (19/20).

None of the interviewees reported taking *H. crenata* together with other medication. Regarding side effects, one participant reported that the plant cannot be taken every day because it might cause some toxicity.

The average volume of a preparation ingested for each time that the treatment was administrated was 200 ml.

Symptoms	Type of utilization	Frequency of citation*
Pain	Stomach pain	12
	Headache	6
	Menstrual pain	1
Infections	Uterus/bladder infections	1
Flu	Coughs	1
	Fever	4
	Treatment of flu	3
Others	Diuretic and anxiolytic	1
	Worms	3
	“Blood cleansing”	1
	Sinusitis	1
	Use in babies nappies to help calm the baby	1
How they feel after taking <i>H. crenata</i> as medication		
Relieved of symptoms		17
Calmer and less anxious		3
Administration route		
Oral		15
Bath		5

*More than one category can be mentioned by each interviewee

Table 2 - Description of utilisation of *H. crenata* Pohl. Obtained from the combination of the questionnaire results applied in the traditional use survey.

Regarding the preparation method used, seven different methods were reported as described in Table 3, however decoction was the most common. The differences between these methods consisted in variations of temperatures, and the type and concentration of solvents.

Considering the part of plant used, the majority used both leaves and stems and some reported using the leaves and stems together with the roots (3/20). The plant could be prepared using dry (8/20) or fresh (18/20) material. For drying the plant material, it was distributed on a table outdoors, and the place was sheltered from direct sunlight. The material is dry when it is easily broken by hand. After drying it can be stored in brown paper bags or boxes in a cool, dry place shielded from light (5/20). The material can be stored for up to one year.

When enquired how far they travel to collect the plant, 13/20 said that it was not so far, a maximum of 2km walking from their houses.

Methods of preparation	Average of amount of material (g) used for 1l of solvent	Frequency of citation
<i>Decoction</i> – Decoction method consists of placing the plant material in water and heating it for up to 30 minutes	50	11
<i>Infusion</i> – The infusion consists of adding water at approximately 80C° onto the <i>H. crenata</i> leaves and covering it.	23	5
<i>Cold water extraction</i> – <i>H. crenata</i> leaves and stems are slightly crushed and left for 2 hours.	20	2
<i>Cold 15% alcohol extraction</i> – the plant materials are placed in a container with wine and left for 8 days.	7	2
<i>Cold 40% alcohol extraction</i> – the plants are placed in a container with pinga, a Brazilian spirit, and left for 1 day.	7	1
<i>Milk decoction</i> – the plant is placed in a pan with milk and boiled for 5 minutes.	60	2
<i>Syrup</i> – after decoction, it is filtered, sugar is added and it is heated until the sugar has melted.	100	1

Table 3 - Description of method of preparation of *H. crenata* Pohl. Obtained from the combination of the questionnaire results applied in the traditional use survey

2.2.3 Discussion

The primary aim of the traditional use survey presented was to describe how people are using *H. crenata* and for what, to ensure that design of the animal experiment corresponded to the traditional use of this plant. It was found that it has been used mainly to relieve pain and the main preparation used is decoction.

Although not specifically described, the pain relief property of *H. crenata* can also be found in the ethnobotanical study carried out by De Jesus *et al.* (2009). This property was not considered because this research group was focused on anti-ulcer and anti-inflammatory symptoms. Therefore, when the interviewee indicated that the plant was used for stomach pain, it was interpreted as anti-ulcer relief. Their results showed that from 38 people interviewed, this plant was one of the most used against ulcer and inflammation.

Between the pain indications mentioned, menstrual and stomach pain raised the question of whether the plant was able to affect the visceral nociceptive mechanism, and also whether it would have an effect in the central or peripheral nervous system, since it was also indicated for headache and inflammatory pain. For this reason it was decided to perform tests using animal models that would answer these questions. Another hypothesis for this plant activity would be that its extract could be inhibiting COX, since it was mentioned to be effective against fever and some cases of inflammation.

The size of the survey was not large enough to have a confident statistical analysis that would correlate method of preparation and type of use. However, this survey was enough to indicate that the decoction extract was the most recommended to treat pain. This plant is also used to treat worms and coughs using a modified version of the decoction extract, where for treatment of worms milk is used as solvent instead of water and to treat coughs sugar is added after the decoction process. Different methods of preparation for the same species are reported in Brazil and worldwide, as well as the use of milk for decoction and syrup (Ghedini *et al.*, 2002; Bhattarai *et al.*, 2010).

Traditionally, the motive for adding milk and sugar to remedies is to disguise the smell or the bitter taste of the plant preparation (Iamoni, 1997; Newton, 2012). For this reason, these two methods of preparation were not reproduced in the laboratory.

The anti-worms and anti-cough properties of *H. crenata* are not reported in the survey published by De Jesus *et al.* (2009). As mentioned in the *Hyptis* studies chapter, this is the only ethnopharmacological study reporting *H. crenata* use. However, the utilisation of *Hyptis* to treat these symptoms is reported for other species of this genus (de Albuquerque *et al.*, 2007; Hossan *et al.*, 2009).

The infestation of worms in the human body, especially in children, induces intense abdominal pain (Wardhan *et al.*, 1993; Memon *et al.*, 2009). Considering that the main use of *H. crenata* is for stomach pain, maybe the treatment used is only relieving the pain symptoms caused by the worms and the person would assume that the infection was cured. Or perhaps, the person feeling an abdominal pain could associate it with worms and presume its infestation. These possibilities make the anti-worms indication doubtful and for this reason it is not explored in this study.

Cough symptoms are widely treated with drugs such as codeine which is also used as pain relief medication (Persson *et al.*, 1995; Eccles, 1996). Like in the pain mechanism process, the symptom of cough also involves the activation of A δ and C fibres nociceptors (Kollarik *et al.*, 2007; Lee and Udem, 2008). Therefore, these facts could suggest that the use of *H. crenata* for the cough symptoms may be also connected with pain relief properties.

The results of the survey show that *H. crenata* was given by parents to their children when they had stomach aches, fever and worms. Also the majority of people described that the knowledge about using and preparing the plant was passed down from their parents or grandparents. This implies that the plant could be a possible low toxicity medicine, since this remedy has been “tested” for efficacy and adverse effects through generations.

The fact that most people use leaves and stems instead of roots, gives a positive aspect for this species in relation to the possibility of it being cultivated. Since leaves and stems can be harvested repeatedly from the same plants. The plant can be collected almost any time, only it is not possible when the leaves fall in the dry season. Also the majority of people described that it can be collected any time during the day, which suggests that the active compounds are relatively stable, since studies have shown that in some plant species variation occurs during the day in the quantity and quality of

compounds in the leaves due to the different amount of sun light exposure (Gobbo-Neto and Lopes, 2007).

The majority of people use the material when it is fresh, however it is possible that this could be due to the availability of *H. crenata*, because for the majority of users the plant grows nearby their homes. Due to the availability of *H. crenata* and that it is a weed that grows fast, for most users there is little need to dry and store the plant, even though some of them are aware that this is feasible as described by one of the interviewed who stores it for a period of one year. However, she emphasised that it is important to store it protected from the light, an indication which could suggest that some of *Hyptis* compounds might disintegrate with exposure to sunlight after the plant is harvested.

Although this plant is known in Brazil as “Hortelazinha” meaning “little mint”, the plant is not usually taken as the common mint tea, it is only consumed as a medication. From the interviews it does not seem to have any addictive properties, since the users do not feel the need to have it as a regular drink.

Some users described burning the *H. crenata* material and hanging babies cotton nappies above it to absorb the smoke, claiming that it would make the baby calmer, while others poured the tea preparation in the bath when they had fever or menstrual pain. These uses may indicate that some of the plant compounds could have a role as an anxiolytic agent or a muscle relaxant. There is a possibility that this effect might be caused by the volatile compounds from the essential oils inhaled or absorbed by the skin during the bath or from the baby nappies. The occurrences of essential oils in *H. crenata* have been reported (Pino *et al.*, 2002; Zoghbi *et al.*, 2002; Rebelo *et al.*, 2009) as well as the ability of some *H. crenata* compounds such as 1,8-cineole to exhibit anxiolytic effects and muscle relaxation (Coelho-De-Souza *et al.*, 2005; Satou *et al.*, 2010).

It is possible that the relief of the symptoms experienced by the users might not be due to the *H. crenata* treatment but could be influenced by the placebo effect. Placebo factors are reported to have influence in the physiology of a person taking a preparation with no therapeutic component (Price *et al.*, 2008). Therefore, the results of this traditional use survey study need to be interpreted cautiously, it still cannot be used to indicate the effectiveness of the use of *H. crenata* species as a medication. For this it would be necessary to carry out a pharmacological study evaluating the effect indicated. In addition, the fact that interviewees have not reported toxic effect using the plant does

not mean that there is no toxicity. In order to clearly demonstrate that *H. crenata* treatments would be safe and effective to use, it would be necessary to carry out a detailed test of the toxicity of the preparation to be used and clinical trials.

2.2.4 Conclusions

The survey carried out with *H. crenata* users shows that this plant is mainly used for pain relief and the parts used are the leaves and stems. The plant is only used as a medication and is prepared using seven different methods. The variation of methods of preparation consists of changes in temperature and in the type and concentration of solvents. The decoction extract method is the most used.

The type of pain treated with *H. crenata* is headache, stomach and menstrual pain. Therefore, it might indicate that the compounds present in the plant induce central and visceral antinociception.

The indication of anti-inflammatory and anti-fever effects raises the possibility of the plant compounds having effects on chemicals messengers such as prostaglandin. The plant compounds might induce cyclooxygenase inhibition, lowering fever and inflammation symptoms.

2.3 Chemical Analyses of Compounds from *H. crenata* and Preparation of Extracts

Since the survey indicated that the main use of *H. crenata* is for pain relief, the next step of this project was to carry out an investigation that would test the antinociceptive activities of the plant.

Before deciding which type of preparation to use it was important to assess if the quality and quantity of volatile and phenolic compounds from *H. crenata* would vary according to the different temperatures, types and concentrations of solvents used. Therefore, apart from the milk extraction that was indicated for worms and syrup for cough, all other extraction methods mentioned in the survey were reproduced in the laboratory and analysed by GC-MS and HPLC.

In addition a preliminary HPLC analysis was carried out to investigate if salicylic acid would be present in the *H. crenata* sample. Salicylic acid is a phenolic acid and is present in plants due to environmental stress conditions (Borsani *et al.*, 2001).

Pharmacologically, salicylic acid is widely known for its ability to reduce fever, inflammation and pain. This effect is obtained through inhibition of cyclooxygenase that consequently reduces prostaglandin production (Ekinici *et al.*, 2011).

2.3.1 *Material and methods*

2.3.1.1 Taxonomic identity

The taxonomic identity of the plant species was verified by Professor Heleno Dias Ferreira, with voucher N°29738, and deposited in the University of Goias herbarium. A sample of the plant was also identified and stored in the herbarium of Universidade Federal de Mato Grosso – UFMT, with registration N° 37605.

2.3.1.2 Plant collection and preparation

The plant material tested in this study was collected in May 2008 under IBAMA personal license n° 24273-1 during the morning at Morrinho's farm, Porto Esperidiao, Mato Grosso, Brazil, which is in the cerrado area. The cerrado is classified as savanna vegetation and it covers 23% of Brazilian territory (Ratter *et al.*, 1997).

Only well-developed plants were selected and the entire plant from the ground upwards was collected (the roots were left in the ground). The plants were not flowering at the time. The material was transported in boxes and left to dry for 3 days in a room exposed to wind circulation; this room was similar to a shed that protected the material against direct exposure to sunlight. The material was spread on tables, the room temperature was approximately 30° C. The indication that the plant was dried was when the leaves and stems could be crushed by hand. After drying, the material was packed into a cardboard box and posted to Newcastle University by Professor Evandro Luiz Dall'Oglio from University Federal de Mato Grosso (UFMT) under agreement between UFMT, SISBIO and Graciela Silva Rocha (Newcastle University), SISBIO registration n° 1995110.

After arrival at Newcastle University, the dried *H. crenata* leaves and stems were reduced to powder in a mill (Cyclotec 1093/ mesh 0.5mm) and stored in a freezer at -24 °C.

2.3.1.3 Methods of extraction reproduced in the laboratory

From the traditional use survey, five different methods of extraction were identified which are used to prepare the plant to treat pain related symptoms. These methods were reproduced in the laboratory as described below (see Figure 6) and the amount of plant material used for each preparation followed the traditional use indication as shown in the results section. The temperature during the process of making infusion and decoction and the extract cooling process were monitored and noted in order to have more precise values in relation to the heat fluctuation.



Figure 6 - Different extraction methods reproduced in the laboratory

2.3.1.3.1 Decoction

H. crenata decoction extract was prepared by gradually adding 1 litre of distilled water (25°C) to 50 g of powdered material (dried leaves and stems) in a glass beaker, mixing with a spoon for 5 min in cold water, heating it until it boiled and boiling for 5 min. The decoction was then left for 15 min to cool without covering it. Next, the decoction was

filtered (Filter paper 125 mm 540, hardened ashless, circles, 125mm diameters/Whatman®) and was ready to be chemically analysed.

2.3.1.3.2 Infusion

H. crenata infusion extract was prepared by gradually adding 1 litre of distilled water (80 °C) to 20 g of powdered material (dried leaves and stems) in a glass beaker, covering and leaving for 30 min while monitoring the temperature, filtering and ready to be chemically analysed.

The details of temperature during the process of making decoction and infusion, as well as the cooling processes of decoction are shown in Figure 7.

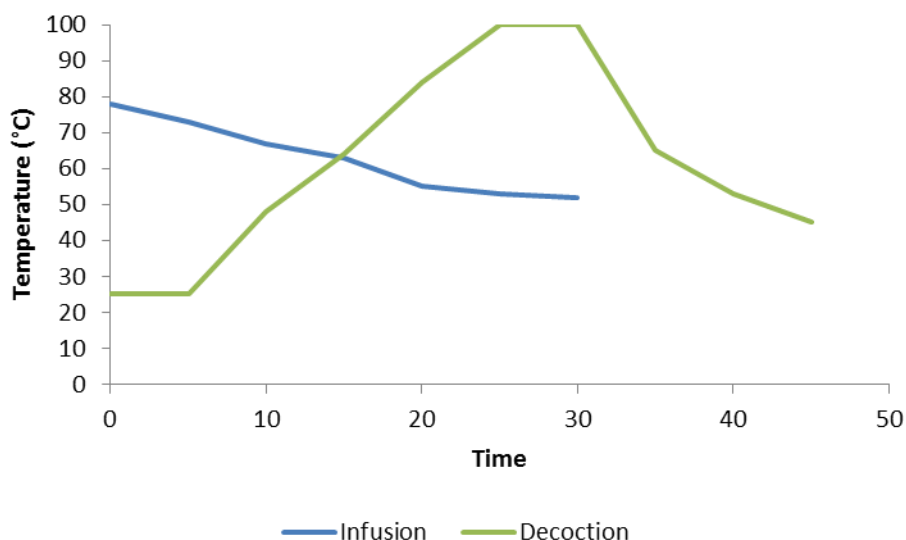


Figure 7 – Details of temperature during the process of making infusion and decoction extract

2.3.1.3.3 Cold extraction (CEwater)

The extract was prepared by gradually adding 1 litre of water (25°C) to 23 g of powdered material (dried leaves and stems) in a glass beaker and mixing with a spoon for 5 min. The solution container was closed, protected from light using household

aluminium foil and left at a temperature of 25°C for 24h (1 day). Then it was filtered and ready to be chemically analysed.

2.3.1.3.4 Cold extraction with water and ethanol (CE15%)

This extraction was to reproduce the traditional preparation with white wine that varies between 10 to 15% of alcohol concentration. This extract was prepared by gradually adding 1 litre of hydroalcoholic solution (15% at 25°C) to 7g of powdered material (dried leaves and stems) in a glass beaker and mixing with a spoon for 5 min. The container was closed, protected from light using household aluminium foil and left at a temperature of 25°C for 144h (6 days). Then it was filtered and ready to be chemically analysed.

2.3.1.3.5 Cold extraction with water and ethanol (CE40%)

This extraction was to reproduce the traditional preparation with “*Pinga*”, Brazilian sugar cane rum, which has an alcoholic concentration of around 40%. Therefore, in the laboratory the extract was prepared by gradually adding 1 litre of hydroalcoholic solution (40% at 25°C) to 7g of powdered material (dried leaves and stems) in a glass beaker and mixing with a spoon for 5 min. The container was closed, protected from light using household aluminium foil and left at a temperature of 25°C for 24h (1 day). Then it was filtered and ready to be chemically analysed.

2.3.1.4 Analyses of quantity of plant material ingested by the people according to different extractions

In order to verify the amount of plant material that has been ingested by people, each extract was freeze dried to quantify the amount of dried weight. 20 ml of each extract were allocated in pre-weighed containers (small bowls made of aluminium foil) and covered with perforated aluminium foil. The containers were freeze dried and the container weight subtracted from the total weight.

2.3.1.5 Analyses of volatile and phenolic compounds by gas chromatography mass spectrometry GC-MS and high-performance liquid chromatography, HPLC.

After preparation, all the five extracts were analysed by GC-MS and HPLC to assess the contents of volatile and phenolic compounds. The analyses were carried out according to the following methods.

2.3.1.5.1 GC-MS conditions

The GC-MS analysis was performed by Paul Donohoe, a technician in the School of Engineering and Geosciences at Newcastle University. GC-MS analysis of the aliphatic or aromatic compounds from the decoction extract was performed on a Hewlett-Packard 7890A GC split/split less injector (280°C) linked to a Agilent 5975C MSD (electron voltage 70eV, source temperature 230°C, quad temperature 150°C multiplier voltage 1800V, interface temperature 310°C). The acquisition was controlled by a HP Compaq computer using Chemstation software, initially in full scan mode (50-600 amu/s) or in selected ion mode (30 ions 0.7 cps 35 ms dwell) for greater sensitivity.

The sample (1µl) in DCM was injected by an HP7683B auto sampler and the split opened after 1 minute. After the solvent peak had passed, the GC temperature programme and data acquisition commenced. Separation was performed on an Agilent fused silica capillary column (30 m x 0.25 mm i.d.) coated with 0.25 µm dimethyl polysiloxane (HP-5MS) phase. The GC was temperature programmed from 50-300°C at 5°C min and held at final temperature for 10 min with Helium as the carrier gas (flow rate of 1 ml/min, initial pressure of 50kPa, split at 30 ml/min). Peaks were identified and labelled after comparison of their mass spectra with those of the NIST05 library if >90% fit or from their elution order from the literature.

2.3.1.5.2 HPLC conditions

The different extractions were analysed on a Shimadzu High Performance Liquid Chromatography (LC-20AD, Diode Array detector SPD-M20A, noise level of 0.6×10^{-5} AU, wavelength range 190-800 nm), Auto sampler Sil-20 AC, Thermo Scientific ODS Hypersil column 250 mm x 4.6 mm, particle size 5 µm. The column (reverse phase) was eluted by using a linear gradient of water (solvent A) and methanol (solvent B), starting with 0% B/100% A (0 min) and increasing to 10% B (10 min), 30% B (20 min), 75% B (35 min), 90% B (45 min), decreasing to 0% B (50 min) and stopping at 65 min. Solvent flow rate was 0.8 ml/min.

2.3.1.5.3 HPLC conditions (salicylic acid analyses)

The decoction extract and a salicylic acid solution (69 µg/2 ml) were analysed in a Shimadzu High Performance Liquid Chromatography (LC-20AD, Diode Array detector SPD-M20A), Auto sampler Sil-20, Column AC Thermo Scientific ODS Hypersil Dim

250 mm x 4.6 mm particle size 5 µm. The column was eluted by using a linear gradient of water + 2% acetic acid (solvent A) and acetonitrile (solvent B), starting with 0% B (0 min) and increasing to 15% B (30 min), 50% B (50 min), 100% B (55-60 min), decreasing to 0% B (60-70 min) with a solvent flow rate of 0.8 ml/min (Kim *et al.*, 2006).

2.3.2 Results

Through GC-MS analysis it was observed that the different extractions have a variation in the type of compounds contained in the samples and also in their concentrations.

The major constituents present in these five extractions were camphor and 1.8-cineole eucalyptol, as shown in Table 4. Also, some other not identified compounds (ONI) were observed. The CE40% extraction was the sample that had the highest ONI, however the maximum concentration reached for any of these compounds was 3.2% and in total these compounds represented up to 19% of the sample. In relation to other extractions, it was observed that the ONI were less than 2% and in total comprised no more than 6.5% of volatile amounts in the samples.

These analyses also showed that the decoction extract has more variety of volatile compounds than the others. The CE40% showed an additional compound, the other extracts only contained camphor and 1.8-cineole in their volatile composition. These two compounds were present in all extracts. Camphor was the major compound in all samples. In relation to the comparison of variation in concentration of camphor and 1.8-cineole; despite the concentration of plant material for preparing the extractions being different (see material and methods) the percentage of total composition of each extraction sample showed that the amount of these volatile compounds was influenced by the temperature and concentration of alcohol. Increased temperature drastically decreased the amount of 1.8-cineole in the decoction (4% of total) compared to cold extraction (24% of total) and infusion (21% of total) that was prepared with low heat. On the other hand, for camphor the % of total component in the sample was higher (84%) in the decoction than for other samples. Another observation was that although the amount of plant material used to prepare the alcoholic extractions were exactly the same, the concentration of each volatile compound increased when the alcohol concentration increased, when comparing CE15% with CE40% as shown in Table 4 and Figure 8.

Extraction	Compounds	% of total	Peak area	Area / mg dry matter (1 ml of extraction)*
Infusion	1.8-cineole (eucalyptol)	21	7770136	3700065
	Camphor	76	27982728	13325109
	ONI	3	1204517	573580
	Total	100	36957381	17598753
Decoction	1.8-cineole (eucalyptol)	4	428366	93123
	Camphor	84	9059655	1969490
	Borneol	2.4	266221	57874
	Thymol	2.3	250647	54488
	Trimethyltricyclododecane-2,9-diol	1.2	135818	29526
	ONI	6.1	705344	153336
	Total	100	10846051	2357837
CEwater	1.8-cineole (eucalyptol)	24	4587181	2698342
	Camphor	75	13973705	8219826
	ONI	1	185397	109057
	Total	100	18746283	11027225
CE15%	1.8-cineole (eucalyptol)	20	2969450	1562868
	Camphor	74	10954430	5765489
	ONI	6	1418649	746657
	Total	100	15342529	8075015
CE40%	1.8-cineole (eucalyptol)	15	4088946	4088946
	Camphor	53	14219520	14219520
	2- (4 - Methoxyphenyl)-2-(3-methyl-4 methoxyphenyl) propane	13	3439737	3439737
	ONI	19	5221844	5221844
	Total	100	26970047	26970047

ONI=other not identified

* The dry matter values for each preparation is shown in the Table 5

Table 4 - Type and concentration of volatile compounds of decoction, infusion and cold extractions reproduced in the laboratory

The HPLC analyses showed phenolic compounds present in the different analyses at absorption of 280nm. It was observed that the decoction and infusion extractions were similar when comparing the types of phenolic compounds, while the cold extracts were remarkably different (Table 4 and Figure 8). The CEwater and CE15% extractions showed two peaks of compound(s) between 17 and 26 minutes retention time (RT), that

are only present in these extractions. Also CE40% was the only one to contain compounds between 34-43 min RT. This indicates that this extraction selected compounds that have a more non-polar behaviour compared to others, since they are appearing later in this reversed-phase chromatography.

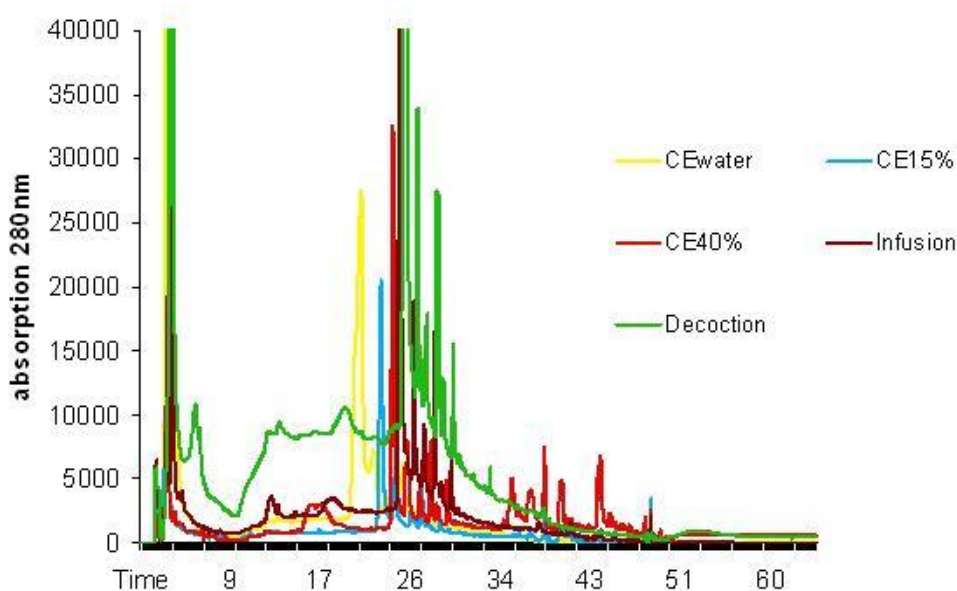


Figure 8 - Comparison of phenolic chemical compounds peaks in the different extractions, according to retention time. High performance liquid chromatography analyses

Regarding the amount of intake of dried extract according to each extract preparation method, the results show that the decoction extract dose is 15 mg/kg body weight (b.w.) being the most concentrated as shown in Table 5.

Extraction	Amount of plant material used	Dried material obtained from 20 ml of sample	Concentration in 200 ml (people treatment)	Concentration in 60 kg (people weight*)
Decoction	50 g/l	91 mg	910 mg	15.2 mg/kg
Infusion	20 g/l	42.1 mg	421 mg	7.0 mg/kg
Cold extract water	23 g/l	34.8 mg	348 mg	5.8 mg/kg
Cold extract 15%	7 g/l	39.6 mg	396 mg	6.6 mg/kg
Cold extract 40%	7 g/l	20.3 mg	203 mg	3.4 mg/kg

*People's weights were estimated as 60 kg (Monteiro *et al.*, 2009), since this was not measured.

Table 5 - Amount of extract dry matter ingested by the people when using the different extractions as treatments.

2.3.2.1 Results of the investigation of salicylic acid in the decoction extract

When comparing the retention time and spectra, the HPLC analyses showed that salicylic acid was not detectable in the *H. crenata* decoction extract (Figure 9). Figure 9A displays the HPLC chromatogram of decoction extract and SA trace. The chromatogram shows that at 38.25 min an overlap occurred between SA and HC decoction, in order to check the similarity between these peaks the absorption spectra was verified. The analyses of these spectra showed that they were different as presented in Figure 9B and Figure 9C.

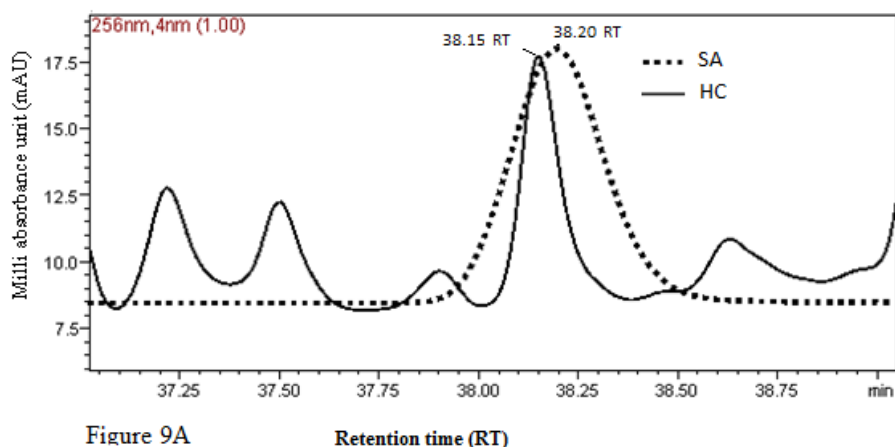


Figure 9A Retention time (RT)

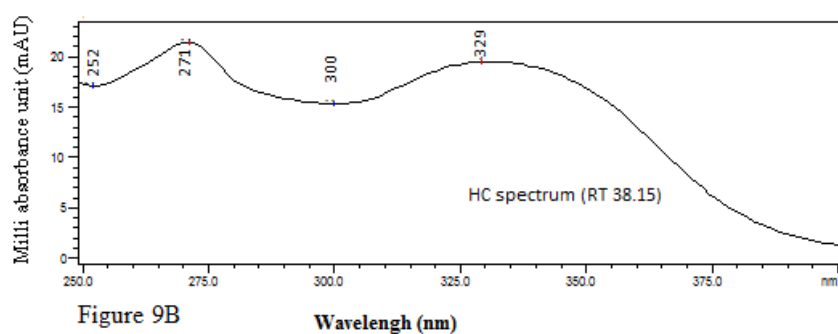


Figure 9B Wavelength (nm)

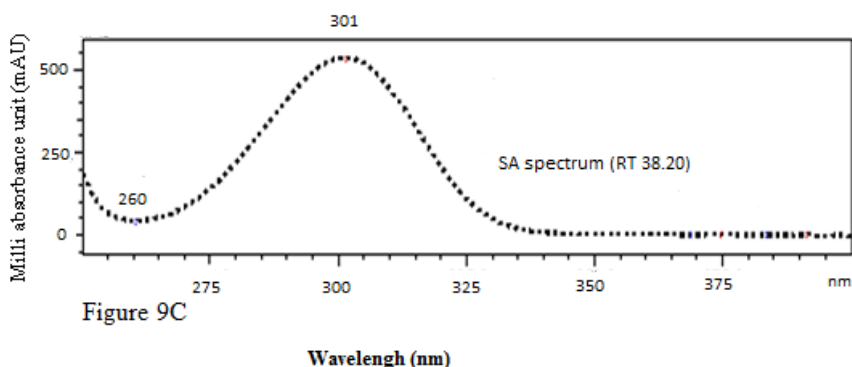


Figure 9C

Wavelength (nm)

Figure 9 – Comparison of spectra from HC compounds and salicylic acid. (A) HPLC chromatogram of *Hyptis crenata* decoction (HC; solid line) and salicylic acid (SA; dotted line) peaks. The chromatogram shows the absorption at 256 nm and indicate that the peak absorption occurs at slightly different retention times (RT). (B,C) Absorption spectra at 250-400 nm for the HC decoction peak at RT 38.15 min (B) and for the salicylic acid peak at RT 38.20 (C). Although the peaks of HC and SA are very close in retention time (A), their spectra are different (B,C), indicating that they are different compounds.

2.3.3 Discussion

The main aim of the experiments presented in this section was to reproduce in the laboratory the extractions that have been described in the traditional use survey and to perform chemical analyses of them. As reported, these extractions were decoction, infusion and three types of cold extractions that used water or its mixture with alcohol at 15% and 40% concentration. The analyses of dried material for each extract preparation showed that the decoction extract, when compared to the others, contains the highest content of dry matter, but it has the lowest amount of volatiles.

As was presented in the results, *H. crenata* extractions showed that temperature and solvent have a significant impact on the quality and quantity of phenolic and volatile compounds present in each preparation. This fact confirms the importance of establishing standard procedures to prepare the plant for consuming as a medication. Otherwise, if the person is not aware of how long the plant should be boiled and, for example, leaves it on the heat longer than recommended, the preparation might suffer changes in the type and concentration of compounds that might have a negative influence in the therapeutic activity, possibly even having adverse effects. The appearance of different compounds influenced by the heat is clearly demonstrated when comparing volatile compounds in the *H. crenata* infusion extract, prepared at 80°C, with a decoction extract that was boiled for 5 minutes. Although in a relatively low concentration the compounds borneol and thymol were found in the decoction but not in the infusion. Few studies are available about the effects of temperature on volatile compound extraction; these are usually not focused on the method for preparing extracts of medicinal plants, but often on using temperature to optimize a method to obtain a certain compound. Examples of this are the work of Xue and Yin (2009) and Yang *et al.* (2009) where the amount of the compound beta-cyclodextrin and zingiberene from the species *Ledum palustre* and *Zingiber officinale* were, respectively, increased or decreased according to temperature variation during the process of sample preparation.

On the other hand, temperature influence on extraction of phenolic compounds is frequently reported. Thus, confirming the results observed for *H. crenata* where the phenolic compounds varied their type according to temperature and concentration of alcohol as a solvent. An example is the results presented by Bucić-Kojić *et al.* (2009) where it was reported that the types and concentration of polyphenols of *Vitis vinifera* L were “highly dependent” on solvent and temperature.

In general, the influence of different types and concentrations of solvents are usually part of the methods used for medicinal plant investigations involving volatiles and phenolic compounds. However, it seems that there is a lack of awareness regarding the influence of temperature when preparing medicinal plant extract at home, and even in the scientific community. Also there are not substantial data that indicate if the general public have this knowledge. This is alarming, in particular when so many people make use of this type of medication, as was described in the introduction to this thesis.

Among the pharmacological activities reported in the literature on the volatile compounds in *H. crenata* are that the compound 1.8-cineole (eucalyptol) has significant sedating and antipyretic activities, and is associated with inhibition of cytokine production and arachidonic acid in anti-inflammatory effects (Ortiz de Urbina *et al.*, 1989; Juergens *et al.*, 1998). The volatile camphor has been described as an agonist of transient receptor potential ion channel vanilloid subtypes 1 and 3, known as TRPV1 and TRPV3. These receptors are present in the skin, dorsal root ganglion neurons and in the brain. They are Ca²⁺ selective channels, camphor has been described to have the ability to “sensitize” TRPV3 and “desensitize” TRPV1, these receptors being responsible for reproducing pain sensation caused by heat and chemicals (Xu *et al.*, 2005; Vennekens *et al.*, 2008; Bang *et al.*, 2012). The compounds thymol and borneol, only found in the decoction extract, were also described for activating TRPV3 receptors, thymol being a more potent agonist than borneol (Vogt-Eisele *et al.*, 2007). In addition, these compounds are indicated to modulate γ -aminobutyric acid, GABA - A receptors, inducing anxiolytic and sedative effects (Vogt-Eisele *et al.*, 2007).

This is the first time that the compound thymol is reported in *H. crenata* species. Volatile compounds of *H. crenata* have been reported by five research groups (Scramin *et al.*, 2000; Pino *et al.*, 2002; Zoghbi *et al.*, 2002; Rebelo *et al.*, 2009; McNeil *et al.*, 2011). The results of these investigations showed that the type and concentration of volatile compounds varied according to the environment where the plant was collected. For example, Zoghbi *et al.* (2002) examined the essential oils of *H. crenata* in different areas of Brazil. The main compounds observed in the essential oil in the area localised in the state of Tocantins in central Brazil were terpinolene (37.8%) and beta-caryophyllene (9.9%), whereas in the state of Para, in northern Brazil the main components were 1.8-cineole (eucalyptol) (23.9%), borneol (21.8%) and beta-caryophyllene (18.8%). Camphor was the main volatile in the aqueous extracts in this *H.*

crenata study, and it was reported for *H. crenata* species growing in Pará and Pantanal, with concentrations of 11% by Scramin *et al.* (2000) and 17.3% by Rebelo *et al.* (2009), respectively.

One conclusion from this literature is that the volatile compounds of *H. crenata* can be different if collected in a different place and time. This PhD project used *H. crenata* material that was collected once, in May, and in one location. The disadvantage of this procedure is that the chemical profile, mainly the volatile compounds of *H. crenata* described in this project, might not represent with accuracy the composition of the plant produced during the year or in other areas, due to the possibility of influence of environmental factors such as drought, soil and sunlight. However, the decision of testing the material with this single collection was based on the results from the traditional use survey, where the majority of users mentioned that the material can be collected any time during the year, therefore suggesting that the compounds that have variation according to environmental changes, such as volatiles, might not play an important role in the medical effects. Also it would not be advantageous to do pharmacological tests using material tests representing different seasons in the year, when the plant studied is a wild species rather than cultivated. Since the compounds found in a native species might suffer changes when this species will be cultivated, as has been verified in studies that showed this variation in volatile and phenolic compounds (Bhatt *et al.*, 2012). Considering if *H. crenata* shows interesting results that might make it worthwhile to produce a phytomedication, the ideal is that the plant would be cultivated, to meet the demand of its use and avoiding the decline of the native species available.

The traditional use survey also indicated that symptoms such as fever and inflammation might be relieved by the plant extract, suggesting that *H. crenata* compounds might have an effect on reducing prostaglandin production, thus decreasing activity of pain mediators such as bradykinin and histamine (Santos *et al.*, 1998). If *H. crenata* compounds are able to inhibit cyclooxygenase, lowering the amount of prostaglandin, this effect could potentially be caused by salicylic acid because this compound is well known as one of the COX inhibitors. However the results of this chapter showed that salicylic acid is not present in the decoction sample, therefore it may be another compound inducing this effect (Xu *et al.*, 1999).

2.3.4 Conclusions

As described, the main indication of *H. crenata* reported in the traditional use survey was for pain relief, and the extracts used for this indication were reproduced according to the traditional use method in the laboratory.

The chemical analyses showed that the amount and type of volatile and phenolic compounds in the extracts were influenced by the temperature used during their preparation and also for the type and concentration of solvent, in this case alcohol and water.

The decoction was the extract that showed the greatest variety of volatile compounds, some of them, such as thymol and borneol, were only found in this type of extraction. The compounds camphor and 1.8-cineole (eucalyptol) were present in all extractions, and camphor was the major volatile in all the samples.

Finally, *H. crenata* decoction extraction compounds are not identical with salicylic acid.

2.3.5 Implications for the next stage of the project

The next stage of this project was to evaluate the antinociceptive activities of *H. crenata*, since pain relief was reported as the main indication. The decoction extraction that has been used at the dose of 15 mg/kg body weight, was the method chosen to prepare the pharmacological treatments to be tested. This was the most common method used in the traditional use survey. Furthermore, this extraction method exhibited a more diverse composition, which showed a range of different types and concentrations of phenolic and volatile compounds when compared to other methods. However, for the pharmacological study the volatile compounds were not tested because the decoction extract, the most used method by the *H. crenata* users, gave the lowest concentration of volatile compounds per mg extract dry material compared to other methods used, indicating that the volatiles were not important for the effect. Additionally, these types of compounds are difficult to evaluate due to their variability that occurs under environmental conditions where the plant grows. Therefore the *H. crenata* treatments tested in this study did not contain the volatile compounds mentioned earlier in the decoction results presented. As the treatments tested in this study were lyophilised, this process removed those volatile compounds. In the results of the traditional use survey there are indications that the plant is used for stomach pain, menstrual pain, headache,

fever and uterus or bladder inflammation. Therefore, we could propose that the decoction extract might have an effect on physiological pain, inflammatory pain and/or visceral pain. To answer if *H. crenata* would be effective against pain caused by chemical stimuli like in the indigestion cases, the acetic acid test inducing writhing was used. In this test an animal model was used which is described to mimic the visceral pain suffered by humans. In addition, we also investigated whether *H. crenata* was an inhibitor of the peripheral nociceptive system. In order to test this hypothesis, the Hargreaves test was used. By using this test we could verify whether the extract was affecting the activation or transmission of the thermal nociceptive system.

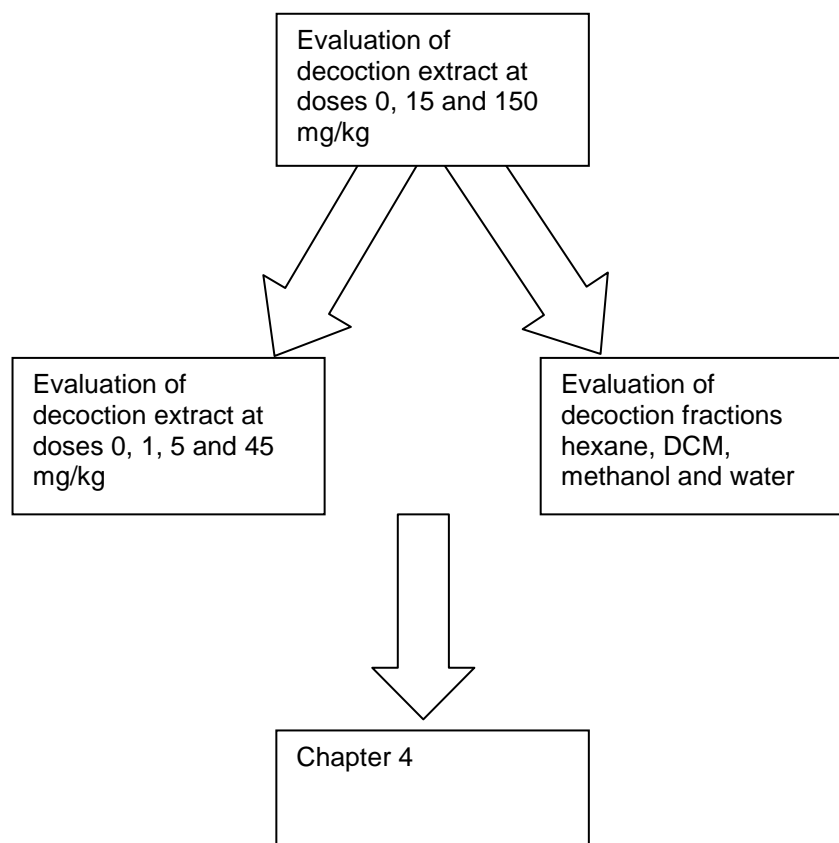
The use survey also indicated that symptoms such as fever and inflammation might be relieved by the plant extract, suggesting that *H. crenata* compounds might have an effect on reducing prostaglandin production, thus decreasing activities of pain mediators such as bradykinin and histamine (Santos *et al.*, 1998). If *H. crenata* compounds are able to inhibit cyclooxygenase and consequently lower the amount of prostaglandin, this effect cannot be caused by salicylic acid, which is well known as one of the COX inhibitors (Xu *et al.*, 1999), because salicylic acid was not detected in the decoction sample.

Furthermore, the fact that this plant was mentioned as anti-inflammatory could indicate that it has a COX inhibition effect. In order to answer these questions the COX inhibition assay was used to predict the levels of prostaglandin and chemical analysis of decoction compounds was carried out and is presented later in this thesis.

**Chapter 3. Evaluation of Antinociceptive Effects of *Hyptis crenata*
Decoction Extract and Fractions Using Hargreaves Test – Acute
Thermal Test**

3.1 Overall procedures

3.1.1 Flowchart



3.2 Introduction

The results obtained from the survey of traditional use of *H. crenata* (Chapter 2) as well as previous antinociceptive studies on other species of *Hyptis* have suggested that *H. crenata* may possess antinociceptive properties, this was investigated both in respect of acute noxious pain and abdominal pain (Bispo *et al.*, 2001; Lisboa *et al.*, 2006; Marcal *et al.*, 2006; Menezes *et al.*, 2007; Santos *et al.*, 2007b; Arrigoni-Blank *et al.*, 2008; Franco *et al.*, 2011; Raymundo *et al.*, 2011). The Hargreaves test was the method chosen to examine whether *H. crenata* decoction would be able to inhibit pain caused by a noxious stimulus, applied at the surface of the body. This is an acute animal model test which uses the heat of infrared light as the noxious stimulus. It has been widely used for evaluating analgesic drugs and for estimating their activities in humans (Hargreaves *et al.*, 1988). The main advantage of Hargreaves compared to other tests, such as hot plate, is that the animal is unrestrained while applying the pain stimulus, heat, on the paw. Therefore, using this test the animal might be less stressed, contributing to a more accurate measure of antinociception (Kocevski and Tvrdeic, 2008). The challenge for this experiment, or for any experiment using animals as the subject, is the fact that animals are not able to communicate verbally if they are feeling pain or to communicate the intensity or location of pain. Therefore, behaviour is one of the methods that can be used for interpreting and evaluating pain. In the case of Hargreaves test it is the paw withdrawal, measuring how long they can stand with infrared light application (Kesim *et al.*, 2002).

The Hargreaves test can be comparable with other thermal tests such as tail flick and hot plate. The difference between Hargreaves and these tests is that “Hargreaves heats a specific point in the animal paw whereas these other tests heat both paws at the same time” (Galbraith *et al.*, 1993).

3.2.1 Hargreaves test to evaluate activity of medicinal plants and drugs

There are few articles that reported antinociceptive studies of medicinal plants using the Hargreaves test (Feltenstein *et al.*, 2003; Amendoeira *et al.*, 2005; Acosta-Madrid *et al.*, 2009; Kang *et al.*, 2010). However, when a search is made looking for “hot plate” used for evaluation, a larger number of articles appear in the databases Web of Knowledge, Scopus and Medline. It is apparent from the number of antinociceptive studies using hot plate compared to Hargreaves that researchers studying medicinal plant antinociception

have a preference for the hot plate method, or it could be that it is the most accessible compared to Hargreaves test, since any laboratory could possess a hot plate but not specialised test equipment for antinociception plantar evaluation.

On the other hand, if the search is made for “analgesic drugs” using the “Hargreaves test”, this method is more widely used for evaluating new drugs or checking the effects of standard drugs. Some of the drugs evaluated are mirtazapine, acetaminophen, codeine, nalbuphine, tramadol, AM1241, huperzine A, amotrigine, clonidine and dexmedetomidine (Ortiz *et al.*, 2007; Roh *et al.*, 2008; Yanarates *et al.*, 2010; Aggarwal *et al.*, 2011).

Certainly, as with other animal model methods, this test has its disadvantages, such as the interference of the animal skin temperature with the results outcome. To overcome this issue the research needs to minimize factors that would induce changes in the animal’s temperature while being measured (Le Bars *et al.*, 2001).

3.3 Aim of this experiment

The aim of this experiment was to evaluate the antinociceptive effects of different doses of *H. crenata* decoction extract and its fractions by assessing their effects at different times after treatment through the Hargreaves test.

3.4 Material and methods

3.4.1 Preparation of treatments

3.4.1.1 Decoction extract

The method of preparing the *H. crenata* decoction extract studies was the same as described in Chapter 2. The difference was that after the process of making and filtering the decoction extract, it was lyophilised using a freeze dryer machine (condenser temperature = -54°C and the chamber temperature = -25°C). After lyophilisation it was kept in a small sealed container in a freezer (-24°C). When it was needed for the animal experiment, the container was collected from the freezer and left for 60 minutes at room temperature to warm up before opening to prevent condensation on the dry material. It was then measured according to doses and reconstituted in the first experiment with 10 ml water for injection, when evaluating HC 15 mg/kg and HC 150 mg/kg, and for the second experiment was reconstituted with 10 ml of water for injection (98%) +

Polysorbate 80 (Tween) (2%), when evaluating HC 1 mg/kg, HC 5 mg/kg and HC 45 mg/kg.

3.4.1.2 Fractionation of decoction extract

For the study of different fractions, the lyophilised decoction extract was fractionated by hexane, dichloromethane, methanol and water. These solvents were chosen due to their polarity index range being 0, 3.1, 5.1 and 9 respectively (Byers, 2003). The procedure of fractionation was as follows: Firstly, the lyophilised decoction extract was weighed (45 mg) in a plastic centrifuge tube (50 ml). Then, 10 ml of the solvent hexane (HPLC grade CH₃(CH₂)₄CH₃) was added to this material, magnetically stirred for 10 min (motor velocity 2, Ikamag® RH), then centrifuged for 10 min at 4000 rpm to remove any particulate matter, and the solvent phase was transferred to a glass tube and was then dried (see below). The above procedure was repeated with the solvents dichloromethane DCM; (Fisher Chemicals – CH₂Cl₂), methanol (HPLC grade CH₃OH) and water (double purified and deionised by Nanopure Diamon-Barnstead) respectively.

The method for drying the fractions was as follows: The prepared fractions of solvents hexane, DCM and methanol were placed in a sample evaporator (40°C, using nitrogen to dry). Dichloromethane was evaporated in 30 min (4 mg dried yield 8.9% of the original 45 mg), hexane after 50 min (2.1 mg dried yield 4.7%) and methanol at 70 min (14.8 mg dried yield 32.9%). The water fraction was lyophilised in an aluminium foil container (23 mg dried yield 51.1%). In total 43.9 mg of the original 45 mg was recovered. After drying, the glass tubes containing the material for the hexane, DCM and methanol fractions were sealed and stored in a freezer (-24 °C). For the water fraction, after drying, the foil container was sealed by wrapping in Parafilm®, covered with aluminium foil and then stored in the freezer. In order to reconstitute the fractions to be used as treatments for the animals, a tube with 10 ml of water for injection (98%) + Polysorbate 80 (Tween) (2%) was added to each fraction. Firstly, 0.2 ml of Polysorbate solution was added to the fraction tube and stirred with a spatula to dissolve the fraction dried material, and then 9.8 ml of water for injection was added and stirred again and mixed using a vortex mixer.

3.4.1.3 Positive drug control

The positive control used was indomethacin sodium trihydrate (Indocid ® PDA, Ovation), supplied in a vial with 1 mg of powder. The reconstitution of indomethacin was made following its guidelines, which involved adding 1 ml of water for injection to achieve a concentration of 0.1 mg/0.1 ml. Indomethacin was selected as this has been successfully used in numerous studies of the acetic acid and hot-plate tests.

Indomethacin belongs to the group of medicines known as the non-steroidal anti-inflammatory drugs or NSAIDs (Ray *et al.*, 2001; Grahame-Smith and Aronson, 2002). This type of drug inhibits pain by blocking the production of certain prostanoids such as prostaglandin. This blockage is obtained through inhibition of the enzyme responsible for its production, the enzyme cyclo-oxygenase (COX), which synthesises prostaglandin from arachidonic acids (Shipton, 1999).

3.4.1.4 Vehicle control

In the first experiment when *H. crenata* 15 mg/kg, 150 mg/kg and indomethacin were evaluated, water for injection (Hameln Pharmaceuticals Ltd., Gloucester) was used as vehicle. For the second experiment Polysorbate 80 (Tween) in a concentration of 2% was added to the water for injection. The reason for adding polysorbate to the vehicle was that it was used as control for evaluation of the fractions. As mentioned in the fractionation section, the fractions required this non-ionic surfactant in order to be successfully dissolved.

3.4.1.5 Treatment doses and the order of experimentation

The first part of the experiment was to evaluate the antinociceptive effect using the same dose indicated in the traditional use survey (15 mg/kg) and a 10 times higher dose (150 mg/kg) in order to be certain whether there were any effects. The dose of 15 mg/kg was not translated using scaling between species (Green *et al.*, 2009a). The traditional use survey results showed that the average amount of plant material used to prepare a decoction extraction was 50 g per litre of water, and that they consumed an average of 200 ml of this for medication. Therefore, the amount of lyophilised extract was calculated according to the traditional use, with a dose of 15 mg per kg of body weight in the range that would be taken by people (approximately similar mg plant material weight / kg body weight). Humans take approximately 200 ml of an extract containing

910 mg dry matter, which approximates to 15 mg/kg (p.o.). For a 25 g mouse the dose administered was 0.38 mg reconstituted in 250 µl (10 ml/kg b.w.) for the low dose and for the high dose 3.8 mg in 250 µl.

After the doses of 15 mg/kg (p.o.) and 150 mg/kg (p.o.) complete extract were shown to have effects the next step of the project was to evaluate lower and higher doses compared with 15 mg/kg (p.o.) in order to determine dose dependency and effective threshold dose. These doses were 1, 5 and 45 mg/kg (p.o.). The final stage of this study was to evaluate the decoction fractions. The *H. crenata* decoction fractions administered to the animals had the same concentration of dried material obtained after the fractionation process since it was reconstituted with 10 ml of solution equivalent to 10 ml/kg ingested by the animals. Therefore the fractions doses were: 2.1 mg/kg (p.o.) (hexane fraction), 4 mg/kg (p.o.) (DCM fraction), 14.8 mg/kg (p.o.) (methanol fraction) and 23 mg/kg (p.o.) (water fraction). In other words, each animal will have received the same amount of active ingredient as the 45 mg/kg of crude extract, only separated into the four fractions. As in the fractions treatments the HC 1 mg/kg (p.o.), HC 5 mg/kg (p.o.) and HC 45 mg/kg (p.o.) also had 2% of polysorbate 80 (Tween) when the extract was reconstituted. The practice of adding polysorbate 80 to dissolve extracts has been reported in several studies, and appears to have no effect on the animals (Navarro *et al.*, 2006; Wang and Song, 2006).

The positive control indomethacin (ID) was administered at a dose of 10 mg/kg (p.o.) and for the vehicle the same volume of water for injection was given (10 ml/kg).

There were eight mice in each of the active treatment groups, while the vehicle group was evaluated in two batches totalling 16 mice. Cages were used with eight mice in each cage in order to avoid any group effect or transfer of behaviour between batches of animals. The different treatments were each given to two mice in each cage. Each cage was a separate batch for the purpose of performing the test, with the activities of subsequent batches staggered relative to each other.

All the treatments were given orally by gavage using a mouse-compatible tube.

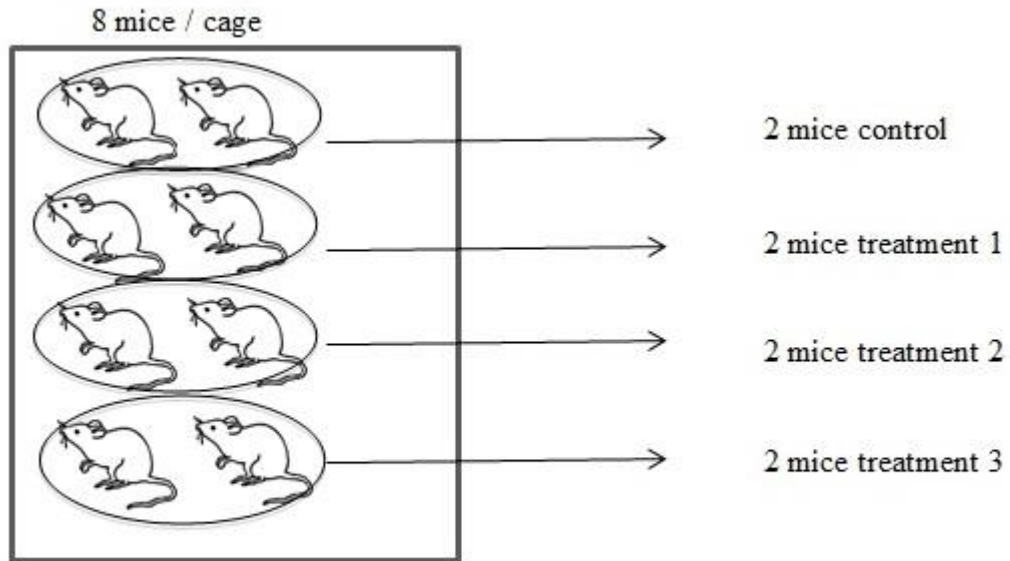


Figure 10 – Diagram illustrating how the mice were housed and the distribution of treatments within a cage.

3.4.2 The Hargreaves test procedure

For the Hargreaves test the thermal nociceptive threshold testing procedure was based on the original protocol published by Hargreaves *et al.* (1988) with some modifications regarding the measurements time point. The reason for this change will be explained in the next section. Foot withdrawal response to thermal stimulation was measured using the Hargreaves apparatus (Ugo Basiles R.L. model 7371), as shown in Figure 11.



Figure 11 - Plantar test instrument (Ugo Basiles), Hargreaves method (Plantar test, 2012)

Each mouse was measured for its baseline nociceptive threshold 24 h before treatment (-24 h baseline). There was no information of when or how long the proposed antinociception for *H. crenata* treatments would occur. For this reason several measurements were taken after the mouse was treated. These measures were taken at 1, 3, 6 and 24 h after treatment.

For the purpose of randomisation, each batch of animals measured in the Hargreaves machine contained a mouse representing a different treatment group. The measurements were taken blinded regarding which treatment the mouse received. The treatments were administered by a technician who performed no further role in the experiment.

The following Hargreaves test procedure was adapted by Professor Paul Flecknell, Dr Matthew Leach and Dr Johnny Roughan from the Comparative Biology Centre of Newcastle University.

Mice were individually confined in Plexiglas chambers (20 cm x 20 cm x 14 cm) on a glass platform. The cut-off time for the equipment was set at 30 s (i.e. the maximum

period of exposure to a heat stimulus). The mobile radiant heat source was placed under the mid-plantar region of the hind paw at an intensity of 50% of maximum heating capacity (190 mW/cm²). The responses (duration until paw withdrawal) for each time point evaluated consisted of taking three measurements from each animal. These measurements were taken from the right and left hind paws, two from the left and one from the right or two from the right and one from the left. Each animal was tested once before moving to the next animal. Measurements alternated between each foot to ensure one foot was not measured more than once within a 5 min period. This interval was allowed to reduce the likelihood of inadvertent alteration in skin nociceptor sensitivity by repetitive testing. Attempts were made to only test mice when stationary and not exhibiting other behaviours (e.g. sleeping and grooming) as this is thought to confound the test, since sleeping animals show attenuated behaviour and grooming mice show relative hypoalgesia (Callahan *et al.*, 2008). The equipment and Plexiglas chambers were cleaned with 10% alcohol solution following each trial to remove urine to ensure that heating remained as consistent as possible and also to avoid the possibility that the urine would induce stress to the next animal to be measured.

The sequence of the measurements at different times performed in the Hargreaves test is displayed in Figure 12.

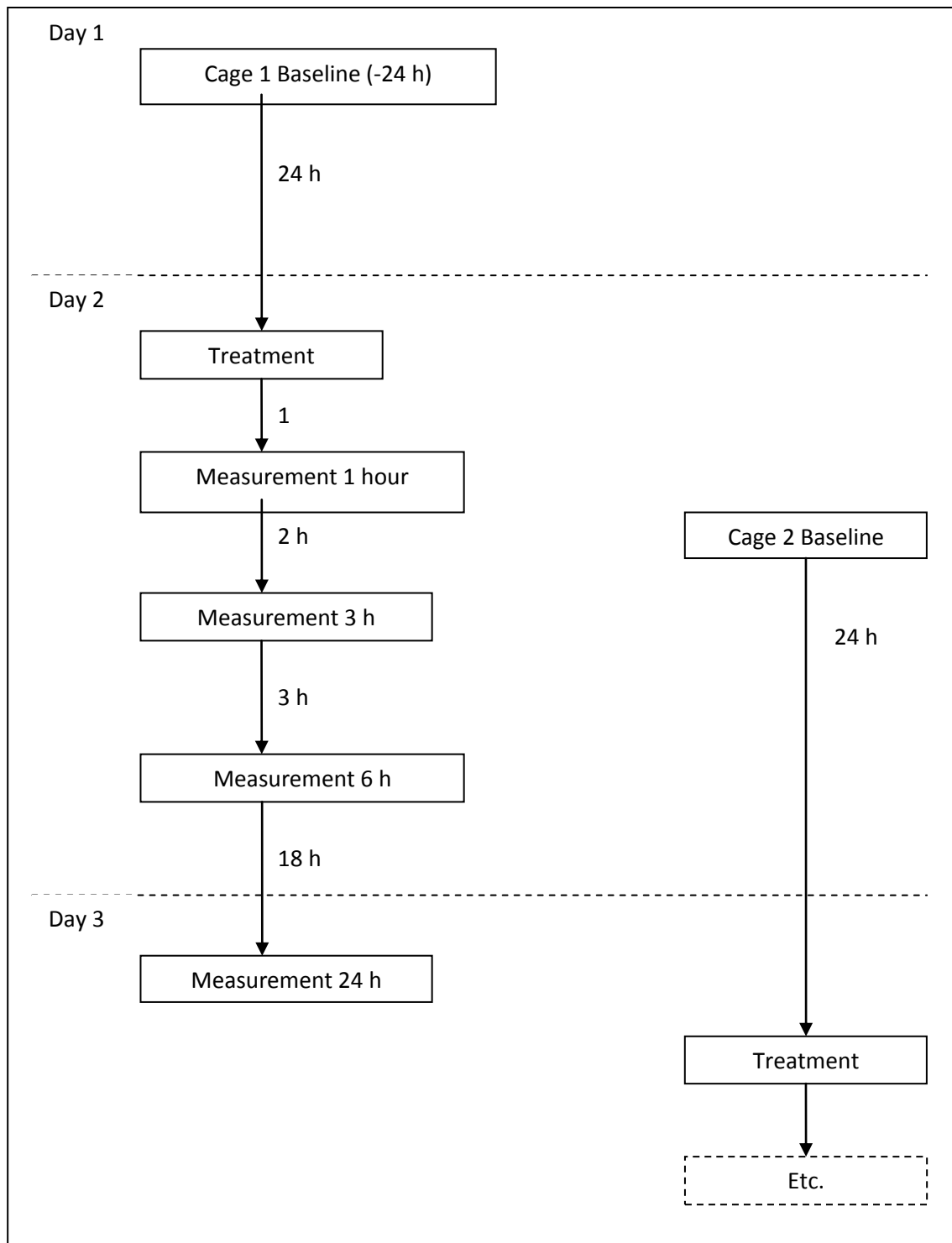


Figure 12 - Treatment and measurement sequence for the Hargreaves test

3.4.2.1 Animals used

The experiment was performed on C57/BL6 mice, from Charles River Laboratories (Margate, UK). This strain was selected for their sensitivity to pain tests and has been

used in the development of several pain models (Anuforo *et al.*; Brierley *et al.*; Harrington *et al.*; Rowley *et al.*; Silva *et al.*; Mirza *et al.*, 2001).

The mice, which were all female and approximately 6-8 weeks old at the time of arrival, were kept for one week to acclimatise before starting the study (room temperature 20°C). They were housed in groups of 8 in plexiglass cages (size: 16" x 11" x 5") and supplied with wood chipping bedding and water and rodent chow *ad libidum*. The mice ranged in weight from 14-25 g. The room temperature was approximately 24° C in the first experiment when testing vehicle, Indomethacin, HC 15 mg/kg and HC 150 mg/kg and 20° C when testing vehicle, HC 1 mg/kg, 5 mg/kg, 45 mg/kg and HC decoction fractions in the second experiment.

The study was carried out under project (PPL N°60/3793) and personal licences (PIL 60/11886) approved by the Secretary of State for the Home Office, under the United Kingdom's 1986 Animals (Scientific Procedures) Act. The premises were also approved and complied with the Code of Practice for the Housing and Care of Animals used in Scientific Procedures, Home Office, 2005. The work was also approved by the local ethical review committee at Newcastle University.

3.4.2.2 Statistical analysis

The mean of the three responses on the hind paws were calculated for each mouse to give a representative nociceptive threshold at each data time point, which were: 24h before treatment (-24 h) and 1 h, 3 h, 6 h and 24 h after the treatment.

In order to remove the individual variation in response from the treatment effect, the average of reaction measurements of baselines were subtracted from the reaction data. Because no effect was observed after 24 h of treatments, the average of 24 h before and 24 h after treatment was used as the baseline, and the average of the baseline measures were subtracted from the 1 h, 3 h and 6 h time points. This provides a value of change in response time. Another reason for choosing 24 h after treatment as baseline is that it would be a more reliable measurement, since the mice would be more familiar with the test environment considering that they could learn from the first measurement that they would receive a thermal noxious stimulus when inside the boxes.

The data were transformed using Log10 for normality fitting; Mean \pm S.E.M. were calculated for all results. The results were analysed using SigmaPlot 11.0. The tests

performed were two way repeated measures ANOVA (time-point x treatment) and one way ANOVA (treatment). A value of $P < 0.05$ was considered significant.

3.5 Results

All animals tolerated the acute administration of the treatments well and no immediate adverse response was observed (although see below for later response to indomethacin).

However, the analyses of effect after time of treatment for the experiments, showed that in all 3 sets of tests most of the withdrawal reaction measurements dropped at 3 h after treatment and increased at 6 h after treatment, as shown in Figure 13, Figure 14 and Figure 15, showing unusual pharmacokinetics for time-effect response. Also, the two way repeated measures ANOVA showed that there was not interaction between time and treatment ($F=0.615$, $DF=20$, $SS=0.442$, $P=0.898$). Therefore, it was decided to analyse the average of the values obtained at 1 h, 3 h and 6 h. In addition, 24 h after treatment the withdrawal response had a similar value as the baseline measurements of 24 h before treatment. Therefore it was decided to use the average of 24 h before and after treatment as baseline.

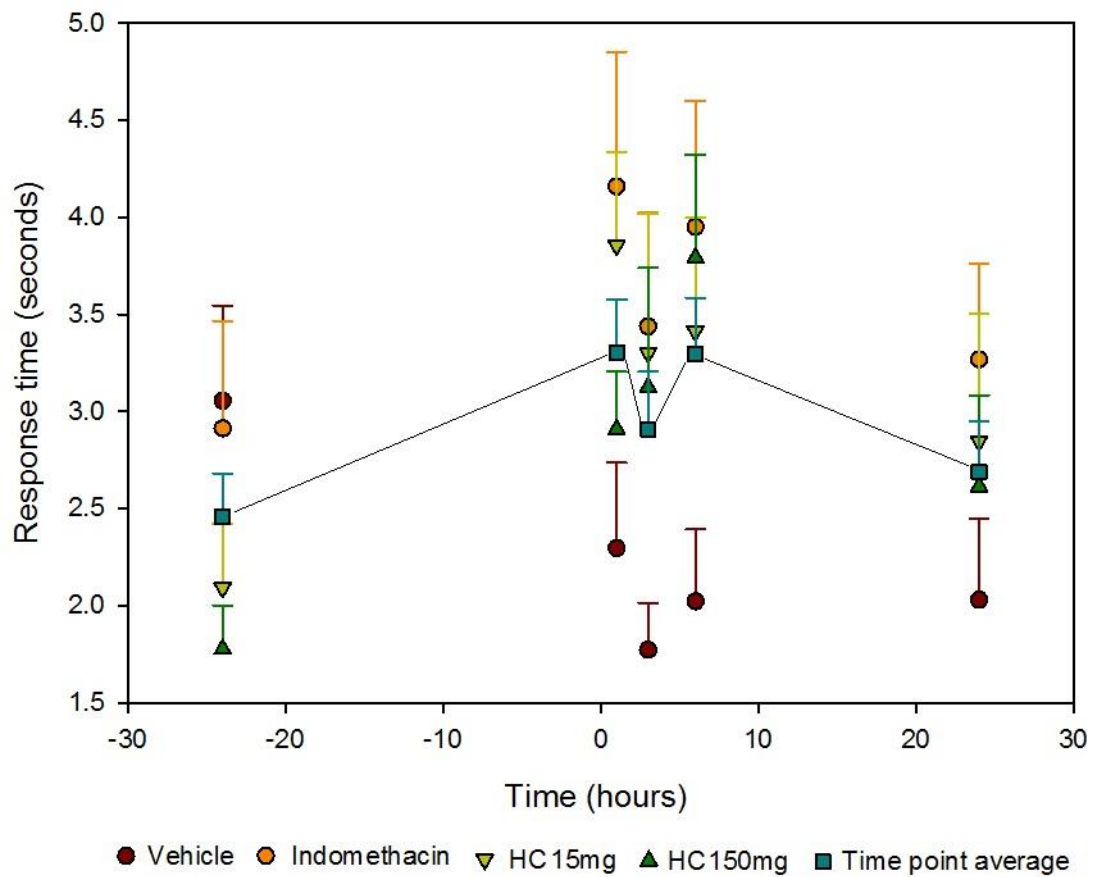


Figure 13 – Withdrawal response delay in the Hargreaves test, evaluation of time-varying response of indomethacin and HC doses. Mean + S.E.M. of data of paw withdrawal response delay plotted against time relative to the treatment with vehicle (p.o.), indomethacin 10 mg/kg (p.o.), *H. crenata* (HC) decoction extract 15 mg/kg (p.o.) and 150 mg/kg (p.o.). All data points are n=8.

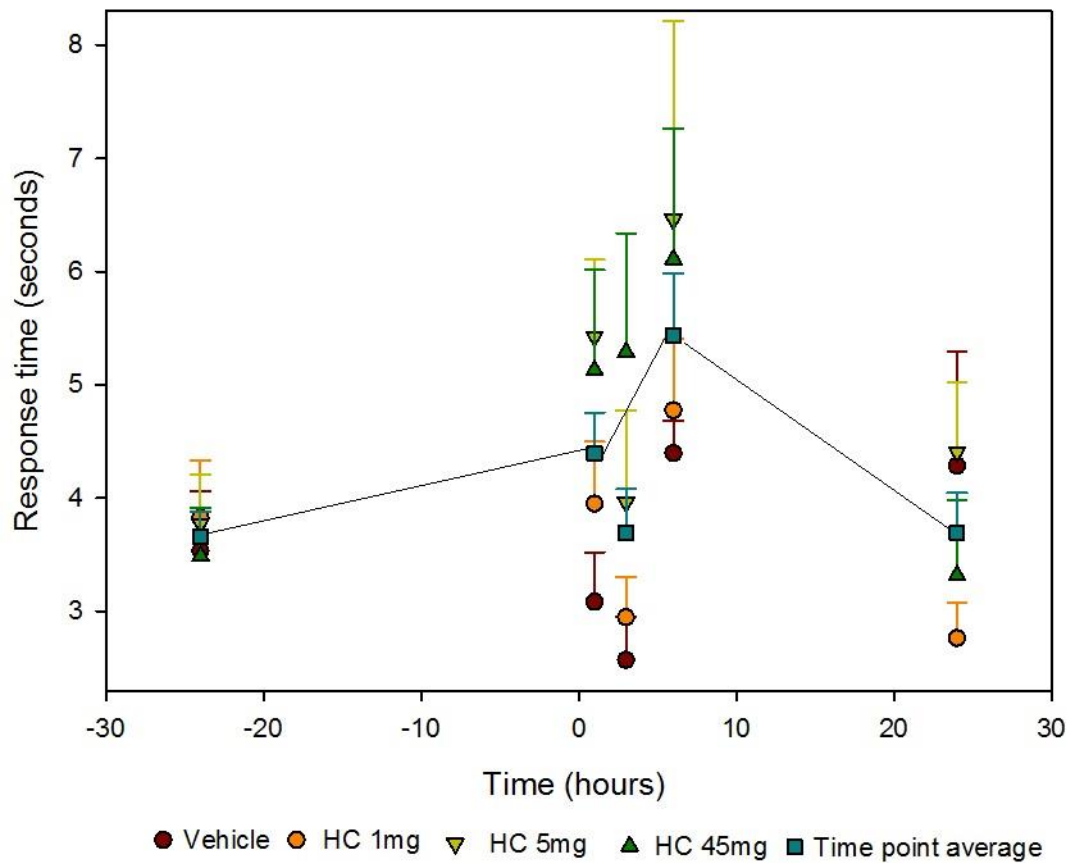


Figure 14 - Withdrawal response delay in the Hargreaves test, evaluation of time-varying response of HC doses. Mean + S.E.M. of data of paw withdrawal response delay plotted against time relative to the treatment with vehicle (p.o.), *H. crenata* (HC) decoction extract 1 mg/kg (p.o.), 5 mg/kg (p.o.), 15 mg/kg (p.o.) and 150 mg/kg (p.o.). All data points are n=8.

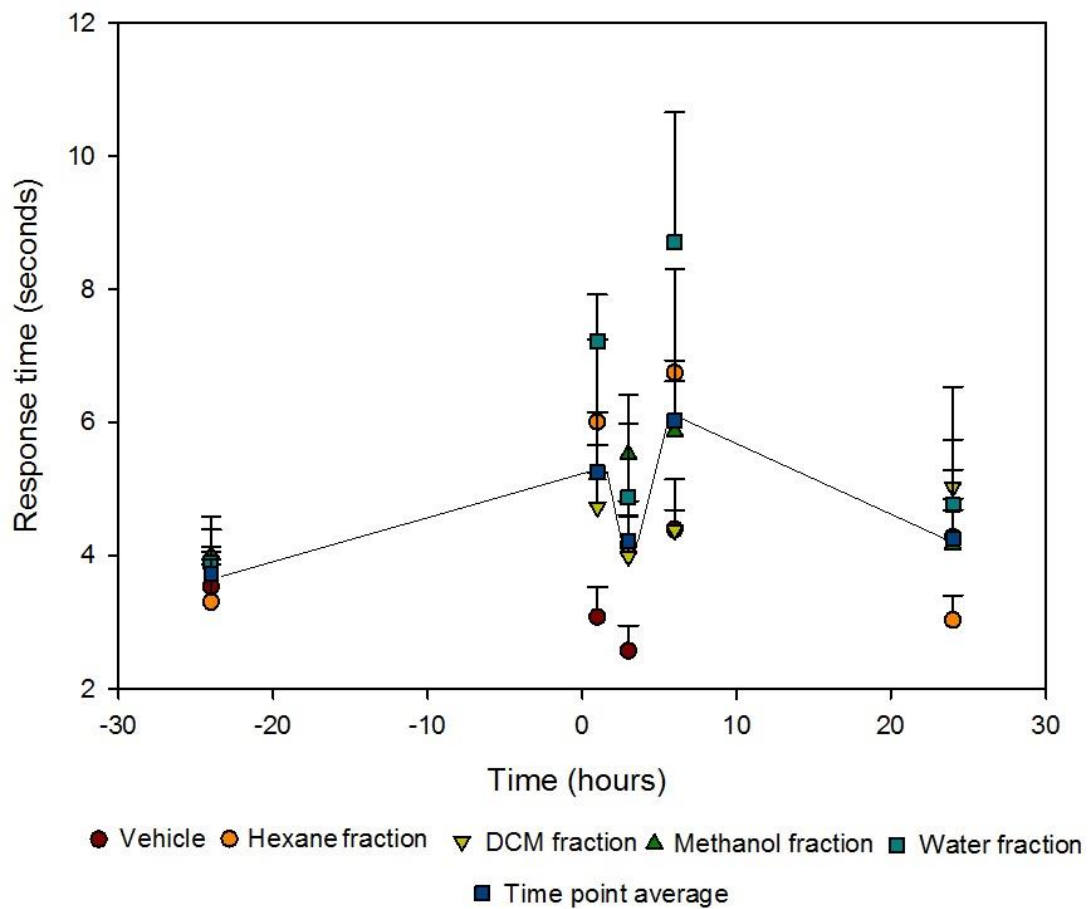


Figure 15 - Withdrawal response delay in the Hargreaves test, evaluation of time-varying response of HC decoction extract fractions. Mean + S.E.M. of data of paw withdrawal response delay plotted against time relative to the treatment with vehicle (p.o.), *H. crenata* (HC) decoction extract fractions (hexane fraction 2.1 mg/kg (p.o.), DCM fraction 4 mg/kg (p.o.), methanol fraction 14.8 mg/kg (p.o.) and water fraction 23 mg/kg (p.o.)). All data points are n=8.

3.5.1 Effect of *H. crenata* decoction extract and its dose-response relationship

The results of the one way ANOVA of groups treated with *H. crenata* decoction showed that the dose of 15 mg/kg (p.o.) and the 10 times higher dose at 150 mg/kg (p.o.) significantly increased withdrawal response delay compared to vehicle. However, indomethacin (10 mg/kg (p.o.)) failed to reach significance level when compared to vehicle (Figure 16, Table 6 and Table 7). The next step of this study was to evaluate dose-response of *H. crenata* doses by testing lower and higher doses compared with 15 mg/kg (p.o.), these doses were 1, 5 and 45 mg/kg (p.o.). The result for this experiment showed significant increase of withdrawal response delay for dose 45 mg/kg (p.o.) when compared to vehicle, but not for the lower doses 1 mg and 5 mg/kg (Figure 17, Table 8 and Table 9). In order to analyse the dose-dependency response for HC decoction doses, the data was plotted and analysed using linear regression calculation. The results of this analysis showed that dose-dependence response occurred from 0 mg to 15 mg/kg, but the response plateaued between 15 mg/kg and 45 mg/kg (Figure 18).

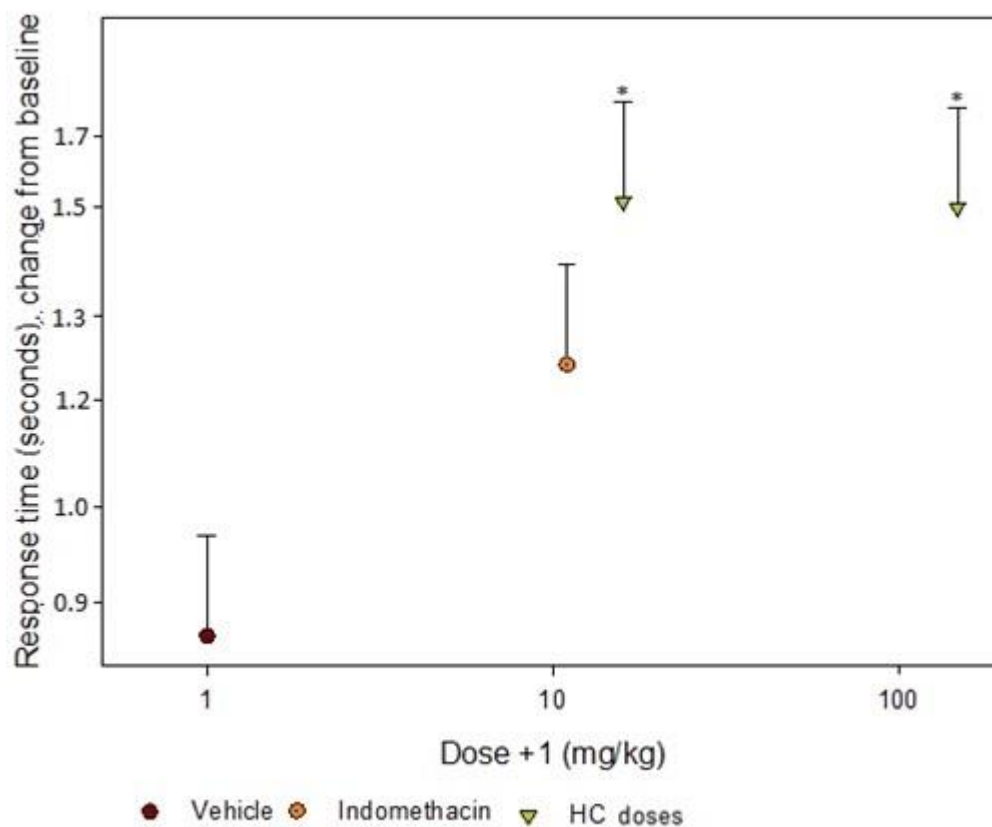


Figure 16 - Withdrawal response delay in the Hargreaves test, evaluation of indomethacin and HC doses response (15 mg/kg and 150 mg/kg). Mean + S.E.M. of sum of paw withdrawal response delay data collected at 1 h, 3 h and 6 h, minus average of 24 h before and 24 h after treatments, animals treated with vehicle (p.o.), indomethacin 10 mg/kg (p.o.), *H. crenata* (HC) decoction extract 15 mg/kg (p.o.) and 150 mg/kg (p.o.). All data points are n=8. * One way analysis of variance multiple comparisons versus control group (vehicle). Holm-Sidak method: Overall significance level < 0.05. (Back-transformed averages of Log10-transformed data, plotted in logarithm scale).

Comparison	Diff of Means	t	Unadjusted P	Critical Level
Vehicle vs. HC 15 mg/kg	0.267	3.080	0.005	0.017
Vehicle vs. HC 150 mg/kg	0.262	3.023	0.005	0.025
Vehicle vs. indomethacin	0.167	1.922	0.065	0.05

Table 6 – Comparison between HC treated groups, indomethacin and vehicle.

Multiple comparisons statistic one way analysis of variance for paw withdrawal response delay data, animals treated with indomethacin 10 mg/kg (p.o.), *H. crenata* (HC) decoction extract 15 mg/kg (p.o.) and 150 mg/kg (p.o.) versus control group vehicle (p.o.). All data points are n=8. Holm-Sidak method: Overall significance level < 0.05. Log10 transformed data.

Source of Variation	DF	SS	MS	F	P	Power
Treatment	3	0.375	0.125	4.147	0.015	0.666
Residual	28	0.843	0.0301			
Total	31	1.218				

Table 7 - Output of one way analysis of variance for paw withdrawal response delay data. Animals treated with *H. crenata* (HC) decoction extract 15 mg/kg (p.o.), 150 mg/kg (p.o.) and indomethacin 10 mg/kg (p.o.) compared with vehicle (p.o.). Holm-Sidak method: Overall significance level < 0.05. All data points are n=8. (Log10 transformed data).

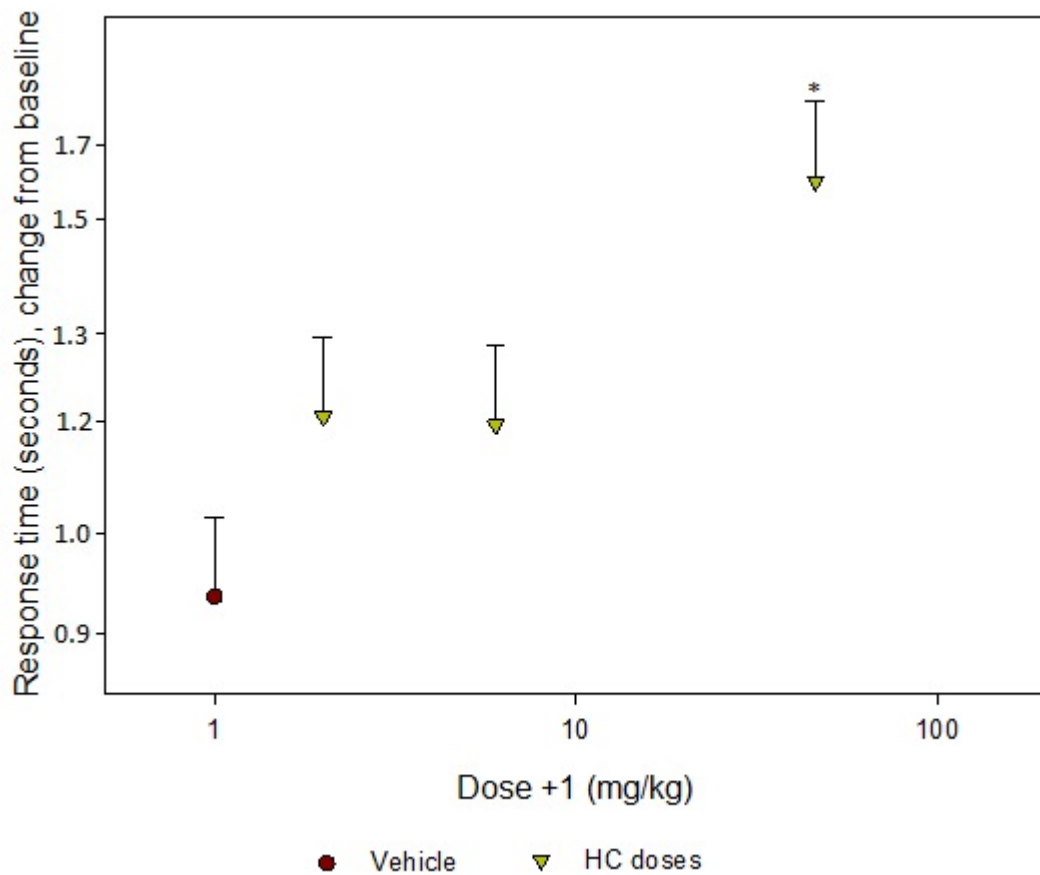


Figure 17 - Withdrawal response delay in the Hargreaves test, evaluation of HC doses response (1 mg/kg, 5 mg/kg and 45 mg/kg). Mean + S.E.M. of sum of paw withdrawal response delay data collected at 1h, 3h and 6h, minus average of 24h before and 24h after treatments, animals treated with vehicle (p.o.), *H. crenata* (HC) decoction extract 1 mg/kg (p.o.), 5 mg/kg (p.o.) and 45 mg/kg (p.o.). All data points are n=8. * One way analysis of variance multiple comparisons versus control group (vehicle). Holm-Sidak method: Overall significance level < 0.05. (Back-transformed averages of Log10-transformed data, plotted in logarithm scale).

Treatment comparison	Diff of Means	t	Unadjusted P	Critical Level
Vehicle vs. HC 1 mg/kg	0.105	1.592	0.123	0.025
Vehicle vs. HC 5 mg/kg	0.101	1.518	0.140	0.050
Vehicle vs. HC 45 mg/kg	0.244	3.680	<0.001	0.017

Table 8 - Multiple comparisons statistic of one way analysis of variance for paw withdrawal response delay data. Analyses of animals treated with *H. crenata* (HC) decoction extract 1 mg/kg (p.o.), 5 mg/kg (p.o.) and 45 mg/kg (p.o.) versus control group vehicle (p.o.). Holm- Sidak method: Overall significance level < 0.05. All data points are n=8. (Log10 transformed data).

Source of Variation	DF	SS	MS	F	P	Power
Treatment	3	0.241	0.0803	4.569	0.010	0.729
Residual	28	0.492	0.0176			
Total	31	0.733				

Table 9 – Output of one way analysis of variance for paw withdrawal response delay duration data. Analyses of animals treated with *H. crenata* (HC) decoction extract HC 1 mg/kg (p.o.), HC 5 mg/kg (p.o.) and HC 45 mg/kg (p.o.) compared with vehicle (p.o.). Holm-Sidak method: Overall significance level < 0.05. All data points are n=8. (Log10 transformed data).

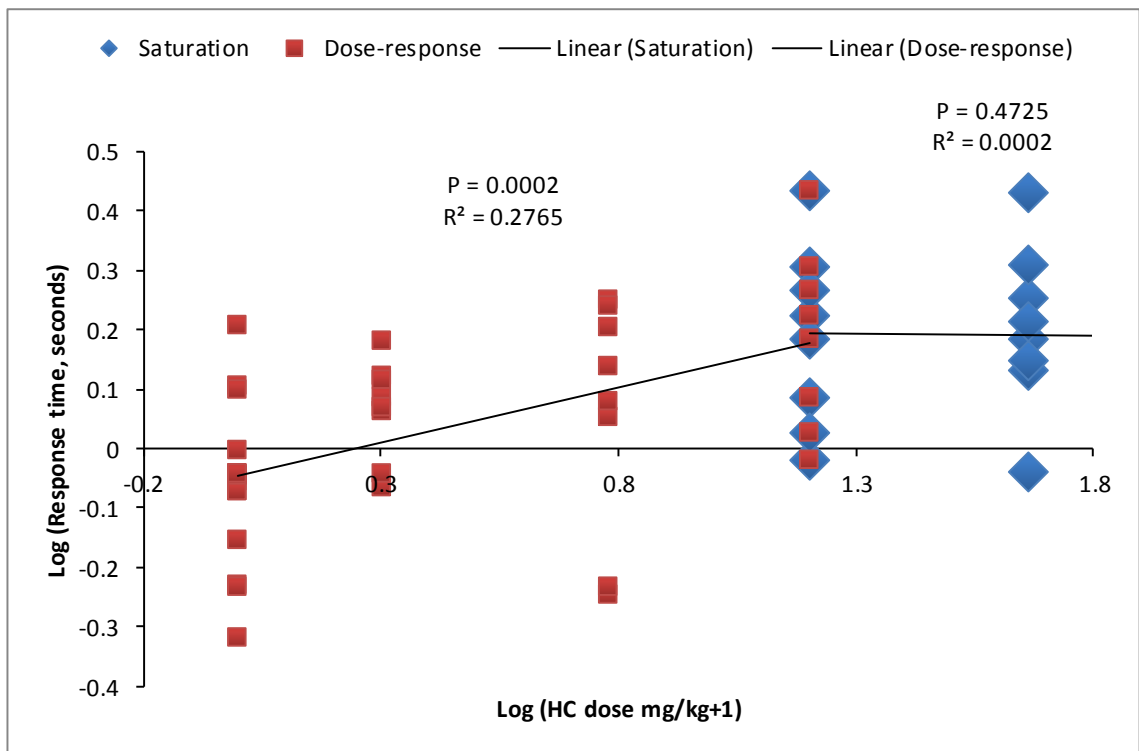


Figure 18 – Dose response of HC treatments during Hargreaves test. Data of paw withdrawal response delay for each animal during Hargreaves test, showing dose response for animals treated with vehicle (p.o.), *H. crenata* (HC) decoction extract 1 mg/kg (p.o.), 5 mg/kg (p.o.) and 15 mg/kg (p.o.) and saturation from the doses 15 mg/kg (p.o.), 45 mg/kg and 150 mg/kg (p.o.). All data points are n=8 except vehicle (n=16). The values show the correlation statistics R2 and significance values for the two phases of the dose-response relationship.

3.5.2 *Effect of H. crenata decoction fractions and comparison between all treatments tested*

This study also evaluated the antinociceptive effect of the HC decoction fractions (hexane fraction, DCM fraction and water fraction). When comparing the effect of the fractions against the vehicle group (n=8), the one way ANOVA results showed that none of the fractions reached significance level (Figure 19 and Table 10), however this results was due to the statistical low power (0.442), because when the analysis was carried out with all HC doses and fractions compared to vehicle (n=16), the results showed there was a significant effect of hexane and water fractions and also it confirmed the significant effect previously observed for HC 15 mg/kg (p.o.), HC 45 mg/kg (p.o.) and HC 150 mg/kg (p.o.) (Table 11 and Table 12).

In order to analyse if there were differences between the effect of the fraction and the HC decoction doses tested, all the data from both experiments were plotted and from the HC doses linear regression the effect between the treatments were compared. The line showed that the hexane fraction was more efficient than indomethacin and that the *Hyptis* decoction extract had the same effect as indomethacin (Figure 20). Although the effects of each of the fractions and the whole extract HC 45 mg/kg (p.o.) are not significantly different from each other regarding increase of withdrawal response delay as shown in the multiple comparison using HC 45mg/kg (p.o.) as a control group (Table 13 and Table 14), the hexane fraction appears to have stronger antinociceptive effect per mg compared with the decoction un-fractionated extract, while this was not the case for any of the other fractions or for indomethacin.

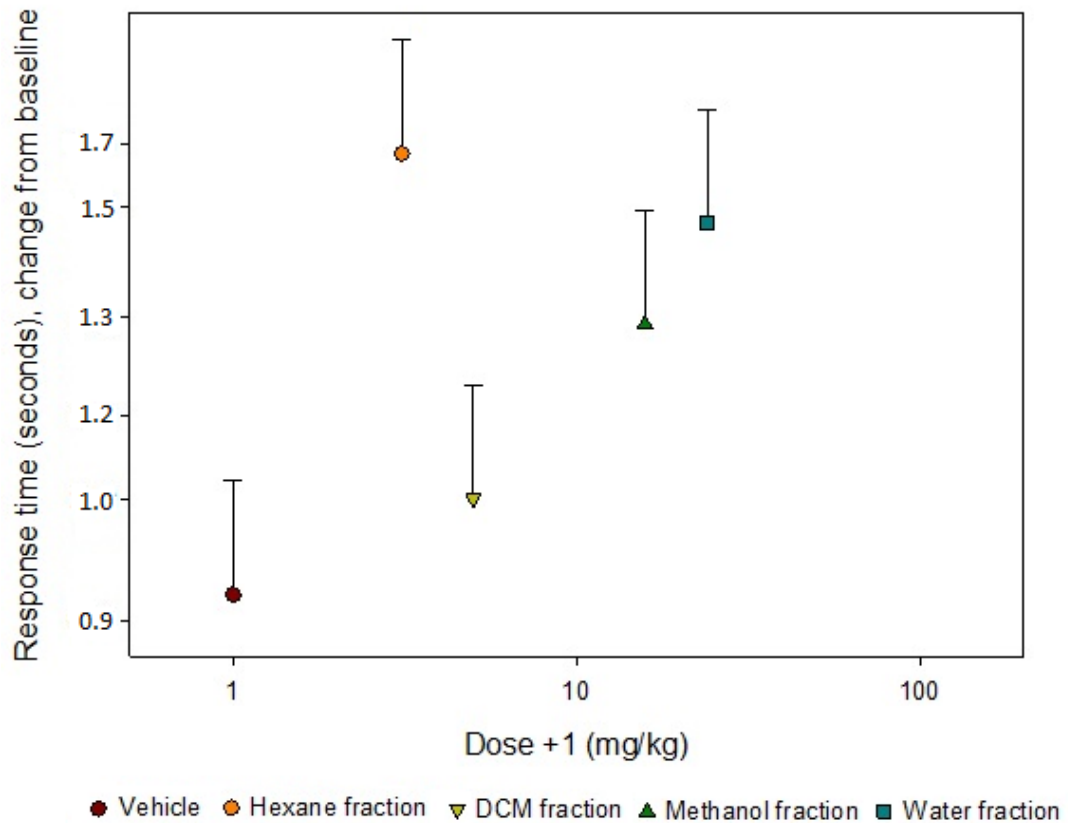


Figure 19 - Withdrawal response delay in the Hargreaves test, evaluation of HC decoction fractions. Mean + S.E.M. of sum of paw withdrawal response delay data collected at 1h, 3h and 6h, minus average of 24h before and 24h after treatments, animals treated with vehicle (p.o.), *H. crenata* (HC) decoction extract fractions (hexane fraction 2.1 mg/kg (p.o.), DCM fraction 4 mg/kg (p.o.), methanol fraction 14.8 mg/kg (p.o.) and water fraction 23 mg/kg (p.o.)). All data points are n=8. * One way analysis of variance multiple comparisons versus control group (vehicle). Holm-Sidak method: Overall significance level < 0.05. (Back-transformed averages of Log10-transformed data, plotted in logarithm scale).

Source of Variation	DF	SS	MS	F	P	Power
Treatment	4	0.389	0.0971	2.629	0.051	0.442
Residual	35	1.293	0.0369			
Total	39	1.682				

Table 10 - Output of one way analysis of variance for paw withdrawal response delay data. Analyses of animal groups treated with *H. crenata* (HC) decoction extract fractions (hexane fraction 2.1 mg/kg (p.o.), DCM fraction 4 mg/kg (p.o.), methanol fraction 14.8 mg/kg (p.o.) and water fraction 23 mg/kg (p.o.)) versus control group vehicle (p.o.). Holm-Sidak method: Overall significance level < 0.05. All data points are n=8. Log10 transformed data.

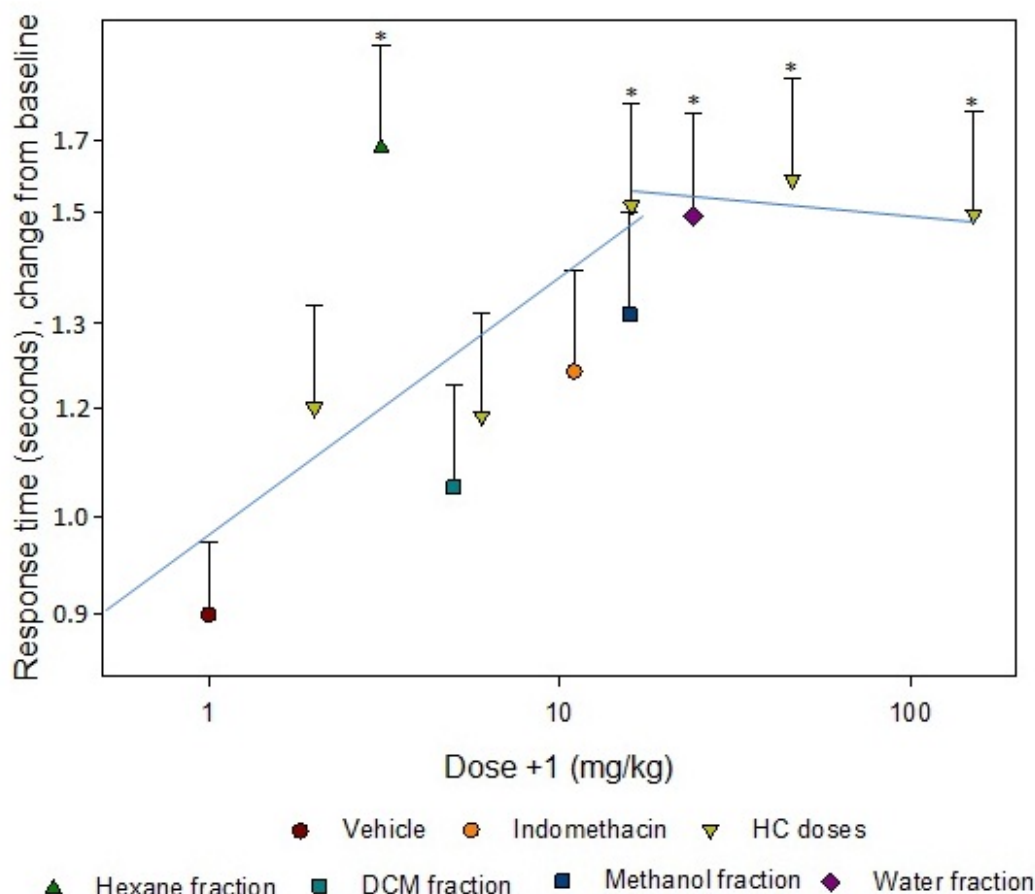


Figure 20 - Withdrawal response delay in the Hargreaves test, dose response of HC doses and decoction extract fractions. Mean + S.E.M. of sum of paw withdrawal response delay data collected at 1h, 3h and 6h after treatment, minus average of 24h before and 24h after treatments, animal groups treated with vehicle (p.o.), *H. crenata* (HC) decoction extract 1 mg/kg (p.o.), 5 mg/kg (p.o.), 15 mg/kg (p.o.), 45 mg/kg (p.o.) and 150 mg/kg (p.o.), its fractions (hexane fraction 2.1 mg/kg (p.o.), DCM fraction 4 mg/kg (p.o.), methanol fraction 14.8 mg/kg (p.o.), water fraction 23 mg/kg (p.o.)) and indomethacin 10 mg/kg (p.o.) compared with vehicle (p.o.). This graph uses the dose response curve previously presented in Figure 18 and indomethacin was plotted as an additional data point in the same coordinates for comparison with *H. crenata* full extract. * One way analysis of variance multiple comparisons versus control group (vehicle). Holm-Sidak method: Overall significance level < 0.05. All data points are n=8 except indomethacin (n=5) and vehicle (n=16) (Back-transformed averages of Log10-transformed data, plotted in logarithm scale).

Treatment comparison	Diff of Means	t	Unadjusted P	Critical Level
Vehicle vs. HC 1 mg/kg	0.125	1.653	0.102	0.017
Vehicle vs. HC 5 mg/kg	0.12	1.588	0.116	0.025
Vehicle vs. HC 15 mg/kg	0.248	3.270	0.002	0.006
Vehicle vs. HC 45 mg/kg	0.264	3.481	<0.001	0.006
Vehicle vs. HC 150 mg/kg	0.243	3.205	0.002	0.007
Vehicle vs. indomethacin	0.147	1.943	0.055	0.013
Vehicle vs. hexane fraction	0.282	3.724	<0.001	0.005
Vehicle vs. DCM fraction	0.0769	1.016	0.313	0.05
Vehicle vs. methanol fraction	0.181	2.390	0.019	0.01
Vehicle vs. water fraction	0.242	3.190	0.002	0.009

Table 11 - Multiple comparisons statistic of one way analysis of variance for paw withdrawal response delay data. Analyses of animals treated with vehicle (p.o.), *H. crenata* (HC) decoction extract 1 mg/kg (p.o.), 5 mg/kg (p.o.), 15 mg/kg (p.o.), 45 mg/kg (p.o.) and 150 mg/kg (p.o.), its fractions (hexane fraction 2.1 mg/kg (p.o.), DCM fraction 4 mg/kg (p.o.), methanol fraction 14.8 mg/kg (p.o.), water fraction 23 mg/kg (p.o.)) and indomethacin 10 mg/kg (p.o.) compared with vehicle (p.o.). All data points are n=8 except indomethacin (n=5) and vehicle (n=16). Log10 transformed data.

Source of Variation	DF	SS	MS	F	P	Power
Treatment	10	0.866	0.0866	2.832	0.004	0.810
Residual	85	2.599	0.0306			
Total	95	3.465				

Table 12 - Output of one way analysis of variance for paw withdrawal response delay data. Analyses of animals treated with *H. crenata* (HC) decoction extract 1 mg/kg (p.o.), 5 mg/kg (p.o.), 15 mg/kg (p.o.), 45 mg/kg (p.o.) and 150 mg/kg (p.o.), its fractions (hexane fraction 2.1 mg/kg (p.o.), DCM fraction 4 mg/kg (p.o.), methanol fraction 14.8 mg/kg (p.o.), water fraction 23 mg/kg (p.o.)) and indomethacin 10 mg/kg (p.o.) compared with vehicle (p.o.). All data points are n=8 except indomethacin (n=5) and vehicle (n=16). Log10 transformed data.

Treatment comparison	Diff of Means	t	Unadjusted P	Critical Level
HC 45 mg/kg vs. hexane fraction	0.00945	0.128	0.898	0.05
HC 45 mg/kg vs. DCM fraction	0.196	2.655	0.009	0.013
HC 45 mg/kg vs. methanol fraction	0.0915	1.242	0.217	0.017
HC 45 mg/kg vs. water fraction	0.0310	0.421	0.675	0.025

Table 13 - Multiple comparisons statistic one way analysis of variance for paw withdrawal response delay data. Analyses of animals treated with *H. crenata* (HC) decoction extract 45 mg/kg (p.o.) and its fractions (hexane fraction 2.1 mg/kg (p.o.), DCM fraction 4 mg/kg (p.o.), methanol fraction 14.8 mg/kg (p.o.) and water fraction 23 mg/kg (p.o.)) versus control decoction extract 45 mg/kg (p.o.). Holm-Sidak method: Overall significance level < 0.05. All data points are n=8. Log10 transformed data.

Source of Variation	DF	SS	MS	F	P	Power
Treatment	4	0.679	0.170	2.726	0.033	0.050: 0.507
Residual	113	7.040	0.0623			
Total	119	7.720				

Table 14 - Output of one way analysis of variance for comparison of paw withdrawal response delay. Analyses of animals treated with *H. crenata* (HC) decoction extract 45 mg/kg and its fractions (hexane fraction 2.1 mg/kg (p.o.), DCM fraction 4 mg/kg (p.o.), methanol fraction 14.8 mg/kg (p.o.) and water fraction 23 mg/kg (p.o.)). All data points are n=8. (Log10 transformed data)

After the Hargreaves test an unexpected fatality occurred in animals treated with indomethacin: 2 mice from the indomethacin group died and 1 was euthanized because it appeared to be unwell. Post-mortem examination of the two mice that were found dead showed that one died from gastric obstruction and another due to liver damage. The mouse that was unwell and was euthanized showed no internal abnormality. The mouse with gastric obstruction died 4 days after being treated, the one that had liver damage died 2 days after treatment and the sickness behaviour of the euthanized mouse was also observed 2 days after indomethacin treatment. No unusual behaviour or adverse events occurred during the Hargreaves test on these animals.

These fatalities only occurred for the animals treated with indomethacin. Under advice of Dr. Robert S Shiel, lecturer of data analyses at AFRD Newcastle University, a Poisson analyses was carried out to predict whether the incidence rate for death events is more than expected by chance. However, due to the small number of occurrences it was not possible to obtain accurate results that would suggest any association of indomethacin as the cause of these deaths Table 15.

<p>Test and Confidence Interval (CI) for Two-Sample Poisson Rates: death, treatment</p> <p>Total Rate of death Occurrences N Occurrence Vehicle 0/8 0.000 Indomethacin 3 /8 0.375</p> <p>Difference = Rate (vehicle) – rate (indomethacin) Estimate for difference: 0.375 95% CI for difference: (-0.799345, 0.0493447) Test for difference ≥ 0 (vs not = 0): Z = -1.73 P-Value = 0.083, Exact Test: P-Value = 0.250* NOTE * The normal approximation may be inaccurate for small total number of occurrences. Analyses carried out with Minitab 16</p>

Table 15 - Poisson analyses for incidence of mouse death.

3.6 Discussion

The aim of this experiment was to verify the ability of *H. crenata* decoction and its fractions to inhibit nociception caused by thermal stimuli. These study results supported thermal antinociception induced by *H. crenata* compound(s) present in the decoction extract and its fractions; hexane and water fractions, although hexane and water fractions only presented significant effect when analysed with a larger number of treatment groups.

This was the first study to report the antinociceptive effect of *H. crenata*. However, this species is not the only one in the genus to be reported with this property. There are reports on three other species (*H. fruticosa*, *H. pectinata* and *H. suaveolens*). Like *H. crenata* these species are described in literature as having traditional use indication against pain and inflammation. These species have also been tested for their antinociceptive properties through pre-clinical trials, the animal models most used in these evaluations were the hot plate test and acetic acid test (Bispo *et al.*, 2001; Lisboa *et al.*, 2006; Marcal *et al.*, 2006; Menezes *et al.*, 2007; Santos *et al.*, 2007b; Arrigoni-Blank *et al.*, 2008; Franco *et al.*, 2011; Raymundo *et al.*, 2011).

Among these antinociceptive studies on *Hyptis* species, *H. pectinata* was reported in five articles, being the most studied in relation to antinociceptive properties. The first study with *H. pectinata* was carried out by Bispo *et al.* (2001) which evaluated an aqueous extract at doses of 100, 200 and 400 mg/kg (p.o.). According to their results the 200 mg dose presented a percentage inhibition of 47.0% and, surprisingly, the 400 mg dose had a lower inhibition, 37.6% compared to vehicle. The lack of dose-dependence observed in this study could indicate that the dose used was very high causing saturation in the effect. A similar lack of dose-dependence is shown in two studies of *H. pectinata* when the doses of 200 mg/kg and 400 mg/kg were evaluated (Lisboa *et al.*, 2006; Arrigoni-Blank *et al.*, 2008). Similar saturation in the effect was seen in the present study with *H. crenata*, but in a lower dose range between 15 mg/kg and 45 mg/kg and with a dose-dependent effect observed at lower doses.

As described in the treatment preparation, the dose of 15 mg/kg that was effective was based on the results from the traditional use survey, following information about the concentration of extract that people take for effective treatment. However, this calculation is without using dose translation from humans to animals. Dose translation

based on body surface area has been suggested by some research in order to establish a dose to be used in a clinical trial when a drug has been tested in animals (Winneke and Lilienthal, 1992; Reagan-Shaw *et al.*, 2008; de la Torre *et al.*, 2009; Green *et al.*, 2009b; Casteleyn *et al.*, 2010). However, in the *H. crenata* study it would be the opposite, because it is the translation from humans to the animal. If using this suggested animal scale conversion the traditional use dose 15 mg/kg would be translated to 187 mg/kg for testing in the animals. The fact that a significant effect was obtained in a much lower dose without using this proposed dose translation means that the result obtained with this experiment would be better than expected by the dose translation proponents. Although this result also indicates that, if it were used, the 187 mg/kg dose would probably have shown the same effect demonstrated by the 150 mg/kg since it appears that the antinociception effect reached a maximum response at this concentration.

There was no interaction between time and the effect measured after treatment. The main reason for this is due to the fact that all treated groups decreased the delay in withdrawal reflex response at 3 h and unexplainably increased the response delay again 6 h after treatment. This drop in response delay was observed not only in one experiment but when it was repeated. Also it was not only observed for treatments of *Hyptis* decoction extract or its fractions, but a similar reaction pattern was observed for the positive and negative control, indomethacin and vehicle. Therefore, it is possible that this change in reaction threshold was a function of the measurement condition at 3 h, perhaps due to the relatively short time (2 h) between the two test times. Thus, the literature was examined to determine if this same event was reported by other researchers. The Hargreaves results reported had a different pattern of time points, which might not be accurate to make a direct comparison. However, the fact that one of these reported results presented measurements with a 2 h interval and did not show a decrease and subsequent increase in response time (Freynhagen *et al.*, 2006), has placed doubt in the supposition that the event observed in the *H. crenata* experiments might be caused by the test and particular time points. Therefore, a reasonable explanation for this event was not obtained.

In general, conclusive details of the pharmacokinetics and pharmacodynamics of *Hyptis* compounds are still unknown. This is an issue which does not just apply for these species but for medicinal plants in general, because most medicinal plant studies are carried out with complex mixtures of compounds. In the case of *H. crenata* this

complexity can be seen in the chemistry analysis section (Chapter 7), where 88 compounds were detected for the *H. crenata* decoction extract. For this reason it is very complicated to decipher and control the metabolism and the action of these compounds in the body. In fact, these diverse compounds in *Hyptis* could perform different functional responses on their targets, for example some of them could be receptor agonists or antagonists. Thus, they could intensify or moderate the antinociceptive effect according to each compound concentration (Rowland and Tozer, 2011). It is possible that increasing the dose of plant extract would increase the exposure of a compound that would inhibit the effect of other active antinociceptive compounds. This could be the case when evaluating *H. crenata* in a higher dose and also this could explain the results observed by Bispo *et al.* (2001) that reported a greater effect of *H. pectinata* treatment at a dose of 200 mg/kg than at 400 mg/kg. Nevertheless, from the present study it is proposed that the maximum response E_{max} for *H. crenata* decoction extract is obtained between 15 mg/kg and 45 mg/kg, since at 45 mg/kg concentration it appears to reach a steady state regarding its metabolism and possibly its elimination (Mazoit *et al.*, 1993).

There are some suggestions from other reports about a possible antinociceptive mechanism induced by *Hyptis* when evaluated by a thermal test. Although these are not detailed descriptions, it has been reported that opioid receptors are involved in the effects of *H. pectinata* essential oils (Arrigoni-Blank *et al.*, 2008). This assumption is confirmed and described in more detail by Raymundo *et al.* (2011) who evaluated *H. pectinata* essential oils treatments using the hot plate test, and suggested that this plant not only has opioid activities but also affects the cholinergic system. This conclusion was revealed by using naloxone and atropine as antagonists before treating with *H. pectinata* extract, observing that the effect of the plant essential oil was attenuated by these antagonists. Interestingly their results showed antinociceptive activities of *Hyptis* essential oil at lower doses (10 mg/kg), as observed for the *H. crenata* extract shown here, although the present study tested an aqueous extract instead of essential oil. Aqueous extracts of *Hyptis* species have also been described by other authors but in much higher doses (200 and 400 mg/kg) and have been described as inducing antinociception through an opioid mechanism, after testing with naloxone as opioid antagonist (Bispo *et al.*, 2001; Santos *et al.*, 2007b).

It is uncertain if the effect of *H. crenata* aqueous extract and its fractions could be involved in the same antinociceptive pathway as described by these earlier *Hyptis* studies. However, it is possible to suggest that the phenolic terpenes found in these other *Hyptis* species, and also mainly observed in the most active *H. crenata* treatment, which was hexane fraction (as shown in the chemistry section, Chapter 7), could be playing an important role in the thermal antinociception effect. However, there is a limit to how comparable it is to analyse results from different plant species, because even if the plant belongs to the same species, the compounds can be variable due to several factors occurring in the environment. When comparing results between different species, it is even more likely that differences will arise from genetic factors. Furthermore, the method of extraction used to prepare the plant treatment will have an enormous influence on the effect obtained due to the selectivity of the type and concentration of compounds. These contrasting effects can be seen in the study by Menezes *et al.* (2007) which evaluated *Hyptis* essential oil of the species *H. fruticosa* using the hot plate test. In contrast with *H. pectinata*, this species was unable to inhibit nociception. Conversely, an earlier study Marcal *et al.* (2006) had shown that *H. fruticosa* can induce thermal antinociception, but when extracted using the solvent ethyl acetate. The explanation for these results could be that ethyl acetate, with a polarity index of 4.4, might be capturing different compounds not present in the essential oil, therefore it would be able to possess antinociceptive effects which were not observed by Menezes *et al.* (2007).

When reviewing these *Hyptis* studies it is notable that none of the groups that have studied *Hyptis* used indomethacin as a control for the thermal test; all of them used morphine as positive control. However, there is uncertainty whether either of these drugs is ideal to be used as the positive control for the thermal test, because a thermal noxious stimulus mainly recruits A δ fibre (McCormack *et al.*, 1998). There are several studies examining whether these drugs would be efficient to reduce pain under an acute thermal experiment condition and the main conclusion is that when these drugs have been shown to be effective it is due to the occurrence of pain conducted by the C fibres that are possibly sensitised by inflammatory agents (Le Bars *et al.*, 2001). For this reason, it is possible that the antinociceptive activity reported for these *Hyptis* studies might also involve anti-inflammatory effects. However, in the present study, if considering the amount of HC dry material injected by the animals, HC hexane fraction was more efficient than indomethacin in inhibiting pain, this effect became prominent when comparing the hexane fraction and other treatments in the linear regression graph.

Overall, the hexane fraction was the most potent inhibitor of pain, because it was administered at a lower dose (2.1 mg/kg (p.o.)). Furthermore, the fact that the ANOVA showed that indomethacin had an effect, but was not significantly different from the vehicle, could indicate that this experiment was mainly evaluating nociceptive effects transmitted through A δ fibres. Therefore, treatments like the hexane fraction and the effective HC doses might be more likely to be A δ fibre inhibitors (Yeomans *et al.*, 1996).

Another puzzling fact involving indomethacin is that in this experiment three animals died and they were all from the indomethacin group. There is no obvious explanation for this event, mainly because the statistical analysis was not able to reveal that the fact that these animals died from only one treatment group would be greater than expected by chance, which would suggest that indomethacin could be associated with these deaths. However, these deaths occurred more than 48 h after the indomethacin intake. Considering that Indocid has a relatively short half-life of 4.5 h (Drug Bank, 2012), theoretically when these mice died they should not be exposed to the administered drug, but it is possible that they suffered side effects caused by an indomethacin metabolite (Rowland *et al.*, 1972). One of these effects could be the gastric blockage observed in the mouse that died four days after treatment. Moreover, another aspect that would increase the correlation of indomethacin treatment with this death is the fact that gastric side effects have been substantially reported for this non-steroidal anti-inflammatory drug (Tomisato and Mizushima, 2003).

Many questions still remain regarding how the antinociceptive effects of the decoction extract or its fractions occur. The Hargreaves results show that some of these ingested compounds are clearly affecting the pain pathway, since the mice treated were able to withstand heating from the infrared light for longer, but it is not possible to predict how and where in the pain pathway the *H. crenata* compounds are acting. These compounds could have their action from the beginning of the thermal pain pathway, impeding depolarisation of nociceptive neurons, or could be affecting the ascending pathway via spinal cord projections to the brain or even blocking the discriminatory pain feeling processed in the cortex or simply intercepting the pain through the descending control (Paxinos, 2004). There are numerous possibilities for the mechanism of action and these increase further when you bear in mind the test treatment contains 16 to 88 compounds in the sample (Chapter 7). An additional factor in relation to these large numbers of

compounds is that they have different concentrations in the sample, making the interpretation of mechanisms even more challenging, because each of these compounds could be performing completely different things, since some of them have a very distinct structure. Also, multiple compounds could be acting on similar functions, thereby having a synergistic effect. Thus, if this synergy was happening, the effect would be that these compounds together would result in a greater antinociceptive action than when fractionated (Wagner, 2011). However, when comparing the decoction extract with the effective fractions it seems that the hexane fraction possessed a greater inhibition of pain than the crude extract. Also it is interesting that the DCM and methanol fractions were not effective. This indicates that the compounds present in these fractions might not be important in the antinociceptive process. Therefore, it is possible to rule out a synergy effect for all decoction compounds together, and propose that there are selective compounds that might be the key protagonist in this antinociceptive event. On the other hand, it is not possible to discard that the effect observed from the hexane fraction is achieved through all compounds acting together. In order to verify this possibility it would be necessary to assess the effect using further fractionation and isolation of compounds. Although this would be interesting to evaluate, exploring this possibility was not a priority for the present study.

The measurement of antinociception obtained through the Hargreaves test is only based on the withdrawal reaction of the mouse's paws. For this reason, it is possible that this type of animal model can present false positive results when the animals are subject to muscle relaxant effects and, despite feeling the pain, they might be slower to move their paw from the noxious stimulus (Le Bars *et al.*, 2001). Such effects are observed through anti-cholinergic drugs, such as the curare derivative compounds (Sterz *et al.*, 1986). Because *Hyptis* has been reported to have some effects as a cholinergic agonist, this would make the muscle relaxant effect less likely (Raymundo *et al.*, 2011). Methods for verifying muscle relaxant effect can be through the rota rod method or through locomotor and hanging behaviour analyses (Budden *et al.*, 1979; Zhang *et al.*, 2011; Klein *et al.*, 2012). In the present study, in order to investigate this muscle relaxant influence, behavioural analyses of mice was carried out before and after administration of treatments. This investigation took place as part of the acetic acid test that will be presented in the next chapter.

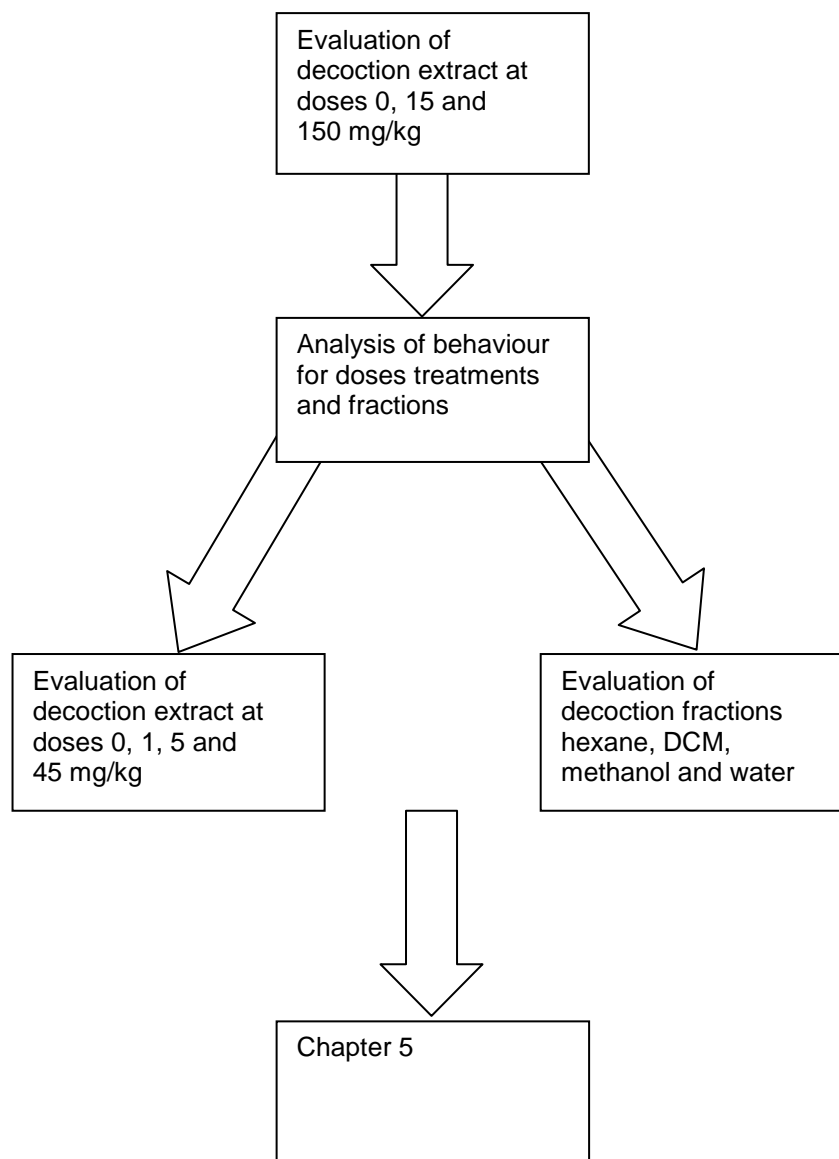
3.7 Conclusions

The study presented in this chapter provides evidence of thermal antinociception induced by *Hyptis crenata* compounds from the decoction extract when administered at doses of 15, 45 and 150 mg/kg (p.o.) and for its 45 mg/kg fractions using hexane and water as solvent. No antinociceptive effect was detected for the decoction fractions extracted with DCM and methanol. There was no interaction between time and the effect measured after treatment. However, the antinociceptive effect terminates within 24 h of treatment. Overall, the hexane fraction possessed the most potent antinociceptive effect, especially when considering that it has the lowest concentration when compared to other *H. crenata* treatments.

**Chapter 4. Evaluation of Antinociceptive Effects of *Hyptis crenata*
Decoction Extract and Fractions Using Acetic Acid Test and
Analysis of Animal Behaviour**

4.1 Overall procedures

4.1.1 Flowchart



4.2 Introduction

Considering that the result of the traditional use survey (Chapter 2) showed that many individuals reported using *H. crenata* for the treatment of stomach pain, it was proposed that this treatment might have an effect on visceral pain. In order to test this possibility, an animal model for visceral pain was chosen, which was the acetic acid inducing writhing test (Collier *et al.*, 1968).

In the viscera the nociceptor endings have been described to innervate the organ walls, the parenchyma, the vessels and the serosal membranes outside the organs. The nociceptor cell bodies are localized in the dorsal root ganglia and nodose ganglia (Gebhart, 2000). Models of visceral pain have been extensively used to evaluate analgesic drugs (Bradesi *et al.*, 2008), these models can involve activation of mechanoreceptors or chemoreceptors that innervate the viscera. Chemical noxious stimuli are specially transmitted by vagal afferents whose cell bodies are localized in the nodose ganglia (Kollarik *et al.*, 2010). An example of mechanoreceptor activation is using physical interventions such as rectal distension with an inflatable balloon to provide an acute stimulus, and chemoreceptor activation can be achieved through acetic acid injection in the animal's peritoneum. The disadvantage of physical intervention with an inflatable balloon is that it requires surgery and does not always permit measurement of free behaviour (Stam *et al.*, 2004). The chemical stimulant model using acetic acid is a more common evaluation method, due to the fact that it only requires the intraperitoneal injection of acetic acid (Martinez *et al.*, 1999). For this reason, and for the fact that this is one of the most widely used tests to mimic visceral pain suffered by humans (Le Bars *et al.*, 2001), it was chosen for evaluation of the visceral antinociceptive properties of *H. crenata* extracts.

The acetic acid model was developed by Koster (1959) for the screening of analgesic drugs and can be used to assess peripherally and centrally acting agents (Tjolsen *et al.*, 1992; Le Bars *et al.*, 2001). Once this chemical noxious agent is injected, it causes tissue irritation when in contact with the peritoneal regions, and this induces the release of pain mediators, such as prostaglandin. Prostaglandin levels have been reported to increase greatly in the peritoneal fluid, particularly after the acetic acid injection, and it has been reported that the levels of prostaglandin 2 (PGE2) were higher than prostaglandin 1 (PGE1) (Collier *et al.*, 1968). The chemosensitivity to acetic acid also involves the proton-sensitive transient receptor potential vanilloid 1 (Sándor *et al.*,

2010). This receptor is considered as a molecular integrator for various noxious stimuli (Bölskei *et al.*, 2005). This pain mediator depresses C fibre transmitting the visceral pain signals (Calvino *et al.*, 1984; Cervero and Laird, 2004). It has also been reported that acetic acid-induced pain signals are dependent on spinal activation of ERK, JNK, p38 and PI3K, and microglia (Pavao-de-Souza *et al.*, 2012). Consequently, this cascade of activation events induces a characteristic abdominal stretching behaviour, or writhing, that was described to be “a pseudo affective reflex response that involves stretches of the torso accompanied by hyperextension of the hindlimbs and concave arching of the back” (Laird *et al.*, 2001). It is this behaviour that is used as an index of pain in the test.

The acetic acid test has been widely used to evaluate the analgesic effects of standard drugs and it has also been extensively used when evaluating medicinal plants, including some species of the genus *Hyptis* which has seven studies reported using this method (Bispo *et al.*, 2001; Lisboa *et al.*, 2006; Menezes *et al.*, 2007; Santos *et al.*, 2007b; Arrigoni-Blank *et al.*, 2008; Franco *et al.*, 2011; Raymundo *et al.*, 2011). In addition, the acetic acid test has been used in studies evaluating analgesic drugs, including common drugs such as paracetamol, gabapentin, diclofenac and nabumetone (Bjorkman, 1995; Seguin *et al.*, 1995; Wetzell and Connelly, 1997; Barden *et al.*, 2004; Hedner *et al.*, 2004).

4.3 Aim of this experiment

The aim of this experiment was to evaluate the antinociceptive effects of different doses of *H. crenata* decoction extract and its fractions by assessing their effects through a visceral model of pain. Before administering the acetic acid test this study also analysed changes of exploratory and motor behaviour of mice after treating with *H. crenata* and its fractions (baseline data).

4.4 Material and methods

The acetic acid test and behaviour analyses (baseline data) were carried out one week after the Hargreaves test using the same mice and also administered the same treatment. The preparation of the decoction extract and its fractions were the same as described in Chapter 2 and Chapter 3.

4.4.1 Treatments and order of experimentation

As in the Hargreaves test described in Chapter 3, the first part of the experiment was to evaluate the dose of *H. crenata* (HC) 15 mg/kg (p.o.), that was indicated in the traditional use survey, and a 10 times higher dose 150 mg/kg (p.o.), together with vehicle and a positive control, indomethacin (10 mg/kg (p.o.)). The next step of this study was to determine the dose-dependency and the baseline behaviour response of *H. crenata* using vehicle (p.o.), 1 mg/kg (p.o.), 5 mg/kg (p.o.) and 45 mg/kg (p.o.). In addition, the four fractions of the decoction extract were examined; hexane fraction (2.1 mg/kg (p.o.), n=8), DCM fraction (4 mg/kg (p.o.), n=8), methanol fraction (14.8 mg/kg (p.o.) n=8) and water fraction (23 mg/kg (p.o.), n=8).

As described in Chapter 3, in order to reconstitute the decoction fractions water for injection was added containing 2% Polysorbate 80, because the fractions required this nonionic surfactant and emulsifier in order to be successfully dissolved. For this reason one of the vehicle groups was treated with water for injection containing 2% Polysorbate 80, as well as the group HC 1 mg/kg (p.o.), HC 5 mg/kg (p.o.) and HC 45 mg/kg (p.o.) that were evaluated in the same batch. In order to check if the polysorbate gave a different effect to the group without polysorbate, a comparison between the two vehicle groups was carried out of the behaviour during the acetic acid test (with and without polysorbate). The results of this analyses showed that there was no difference in the number of writhing occurrences between the two vehicle groups (difference of means (logarithm): 0.197, t: 1.213, unadjusted P: 0.229 and critical level: 0.002).

All the treatments were given orally by gavage using a mouse-compatible tube.

The animals used in this experiment were the same animals used in the Hargreaves test and under the same project license.

In total, this experiment carried out evaluation on 93 mice, and with the exception of the indomethacin (n = 5) and vehicle (n = 16) groups, all other treatments contained 8 animals. The acetic acid test was performed with only 5 mice as positive control, because 3 mice in the indomethacin group died after the Hargreaves test, as was described in Chapter 3.

4.4.2 Recording and analysing behaviour

One week after the Hargreaves test the same mouse used was tested again for evaluation of exploratory and motor behaviours before and after treatments, and after the acetic acid injection. All these mice received the same treatment that was given in the Hargreaves test.

The analysis of exploratory and locomotor behaviour was undertaken to investigate whether *H. crenata* was affecting behaviours that might indicate sedation and/or a muscle relaxation effect. These behaviours were analysed before and after treatments of *H. crenata* doses 0, 1, 5 and 45 mg/kg and decoction fractions (hexane fraction, DCM fraction, methanol fraction and water fraction). The animals were filmed for a 30 min period before the treatment and, after a 30 min wait after the treatment, filmed for another 30 min period. One hour after the treatment was given, the animals received an injection of acetic acid and were filmed for a 45 min period.

The animals were set up in individual clear cages (29.5cm length x 18.8cm width x 13cm height), randomised according to treatments. Each cage had a video camera for recording the mouse behaviour located horizontally in order that all parts of the cage could be seen. The animal cage is shown in Figure 21. The cameras used were Sony Handycam DCR-HC 52, the data were recorded directly onto tape and later converted to an electronic video file to be watched in the Windows Media Player. A randomised code, displayed briefly when the filming of the mouse started, was assigned to identify each mouse to ensure that the measurement was blind to the treatment.



Figure 21 - Cage used for animal behaviour analysis

4.4.2.1 Behaviour data collection periods

For analyses of exploratory and motor behaviour before and after treatments, 10 minutes of footage was analysed immediately before the treatment (from -10 min until 0 min in the timeline recording Figure 22), after treatment during 10 minutes (from 50 until 60 min) and after injection of acetic acid during 20 minutes (from 66 min until 86 min) for 64 mice treated with HC 0 mg/kg, 1 mg/kg, 5 mg/kg, 45 mg/kg and the fractions (n = 8).

After the first experiment it was decided to perform analyses of behaviour before and after treatments, before the acetic acid injection. This analysis was not performed for the groups HC 15 mg/kg, HC 150 mg/kg and indomethacin 10 mg/kg, which were evaluated in the first experiment.

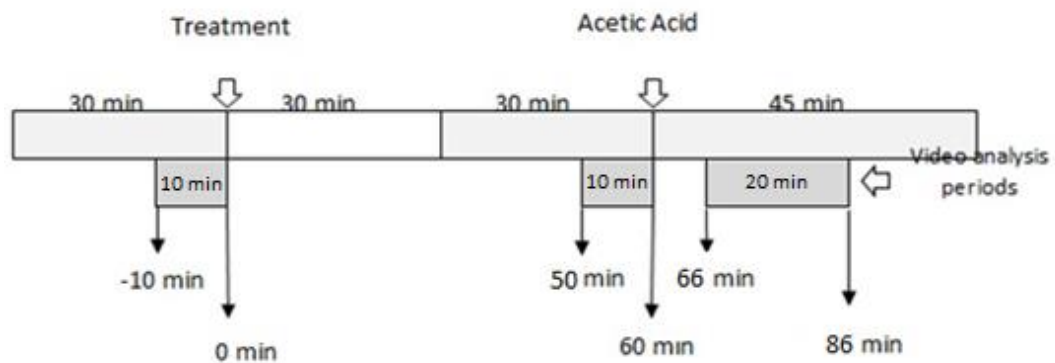


Figure 22 - Timeline of filming and behaviour analysis periods

Each video file was observed and the occurrence of specific behaviour was recorded by using a letter as a code and pressing the letter key on a computer keyboard every time the behaviour occurred. For some behaviour, such as hanging, the duration in seconds was recorded using the recording time showed by Windows Media Player. These codes were typed in an Excel sheet and transferred to SPSS 17 program to be quantified.

For the two periods before and after treatment with HC extracts 10 different behaviours were recorded. These were:

Drinking (number of events): was counted when the animal licked the tip of the water bottle.

Eating (number of events): was counted when the animal ate the food available at the top of the cage,

Sniffing (number of events): was counted when the animal sniffed the cage bedding.

Digging (number of events): was counted every time the animal was observed digging holes in the cage bedding.

Grooming (number of events): was counted when the animal licked or scratched itself (grooming body) or scratched its nose (grooming nose).

Climbing (number of events): was counted when the animal's front paws contacted the cage wall above floor level.

Hanging (duration in seconds): its duration was measured every time the animal climbed to the top of cage and stayed hanging holding the wires with its four paws.

Rearing-up (number of events): was counted when the animal rose on its hind legs.

Walking (number of events): was counted when the animal moved from one location to another in the cage.

Total time stationary (duration in seconds): was counted in seconds, measuring how long the animal stayed stationary; if the animal moved its head or groomed itself it was still considered in a stationary situation.

During the acetic acid test the same behaviours were recorded, but with the addition of writhing and twitching.

Writhing (number of events): was counted when the animal showed a movement of constriction and elongation passing along the abdominal wall, sometimes accompanied by twisting of the body and followed by extension of the hind limbs.

Twitching (number of events): was counted when the mouse had muscle contractions displayed as a tremor.

The total time stationary during the acetic acid test was abbreviated as TSAC, to differentiate it from the total time stationary observed before the acetic acid injection. However as before the acetic acid injection, the total time stationary during the acetic acid test, was also counted in seconds, measuring how long the animal stayed stationary; if the animal moved its head or groomed itself it was still considered in a stationary situation.

4.4.3 Categorisation of behaviour, analyses of data and statistical procedures

Previous studies have characterised different behaviours in respect of the action of drugs and this categorisation has been used in the interpretation of the effects of the *H. crenata* extracts.

The sum of sniffing, grooming and rearing were categorised as (parameter BIS), since the reduction of these behaviours indicate sedation (Rodgers *et al.*, 2000; Aragão *et al.*, 2006; Gomes *et al.*, 2008; Lopes Campêlo *et al.*, 2011). The sum of digging, climbing

and walking were categorised as indicators of locomotor activities and neuroleptic effect (parameter BLY) (Faulkes, 2006; Semler *et al.*, 2011). Total time stationary and hanging were analysed as indicators of motor impairment and muscle relaxant effect. The hanging observed in this study is related to the behaviour observed during the hanging wire test which is used to evaluate muscle relaxant effect (Zhang *et al.*, 2011; Klein *et al.*, 2012), the main difference is that the animals were not forced to hang in the cage wire, they did it freely.

For the acetic acid test evaluation, the behaviours that would indicate exploratory and locomotor activities (parameter ELA) were defined. These behaviours were the sum of sniffing, rearing, climbing, eating, drinking and walking (Baumans *et al.*, 1994; Hawkins, 2002). The behaviours of writhing, total time stationary (TSAC) and twitching were counted separately.

The behaviours BIS, BLY, hanging and total time stationary observed before treatment with *H. crenata* decoction and its fractions were subtracted from those observed after the treatment. Before this difference was calculated a normality test was performed and some of them were transformed using Log10 for normality fitting. The mean \pm S.E.M. of the difference were obtained using SigmaPlot 11.0 and the tests performed were one way ANOVA with multiple comparisons of treatment versus control. A value of $P < 0.05$ was considered significant.

The behaviours observed during the acetic acid test were also analysed with one way ANOVA and the post-hoc test (Holm-Sidak method).

4.4.4 *The Acetic Acid Test procedure*

In this study the acetic acid test was based on the original protocol by Collier *et al.* (1968) with modifications made by the Newcastle pain research group (Flecknell and Roughan, 2004). Sixty min after receiving the treatments, each mouse received an intraperitoneal injection of acetic acid (0.6%, 20 ml/kg b.w.). After the injection the mouse was placed in the cage and filming was started for behavioural analysis, as described in Section 4.4.2.

4.5 Results

4.5.1 Effect of *H. crenata* decoction extract and its dose-response relationship

The first step of this study was to evaluate the antinociceptive activities of HC decoction extract at the dose of 15 mg/kg (p.o.) and a 10 times higher dose of 150 mg/kg (p.o.) against vehicle and indomethacin as positive control. The analysis of writhing during the acetic acid test showed that the HC extracts and indomethacin significantly decreased writhing compared to vehicle ($p < 0.05$) (Figure 23, Table 16 and Table 17). The multiple comparison analyses showed that HC 15 mg/kg (p.o.) and HC 150 mg/kg (p.o.) treatments were as effective as indomethacin in reducing writhing behaviour (Table 18). The fact that indomethacin had shown an effect gave confidence that the experiment procedure was succeeding.

The next step of this study was to evaluate the dose-dependent response of lower and higher doses of HC compared with 15 mg/kg (p.o.), these doses were 1, 5 and 45 mg/kg (p.o.). The result of this evaluation showed that HC 5 and 45 mg/kg (p.o.) significantly reduced writhing when compared with vehicle (Figure 24, Table 19 and Table 20), but no effect on writhing was observed for HC 1 mg/kg (p.o.). The dose response relationship for *H. crenata* decoction extract on reduction of writhing was determined and the correlation analysis showed that there was dose response from 0 mg/kg, 1 mg/kg, 5 mg/kg and 15 mg/kg ($p < 0.0001$, $t = 0.469$, $df = 22$), but that the response plateaued at higher doses of 15 mg/kg, 45 mg/kg and 150 mg/kg ($p < 0.3$, $t = 5.087$, $df = 38$) (Figure 25).

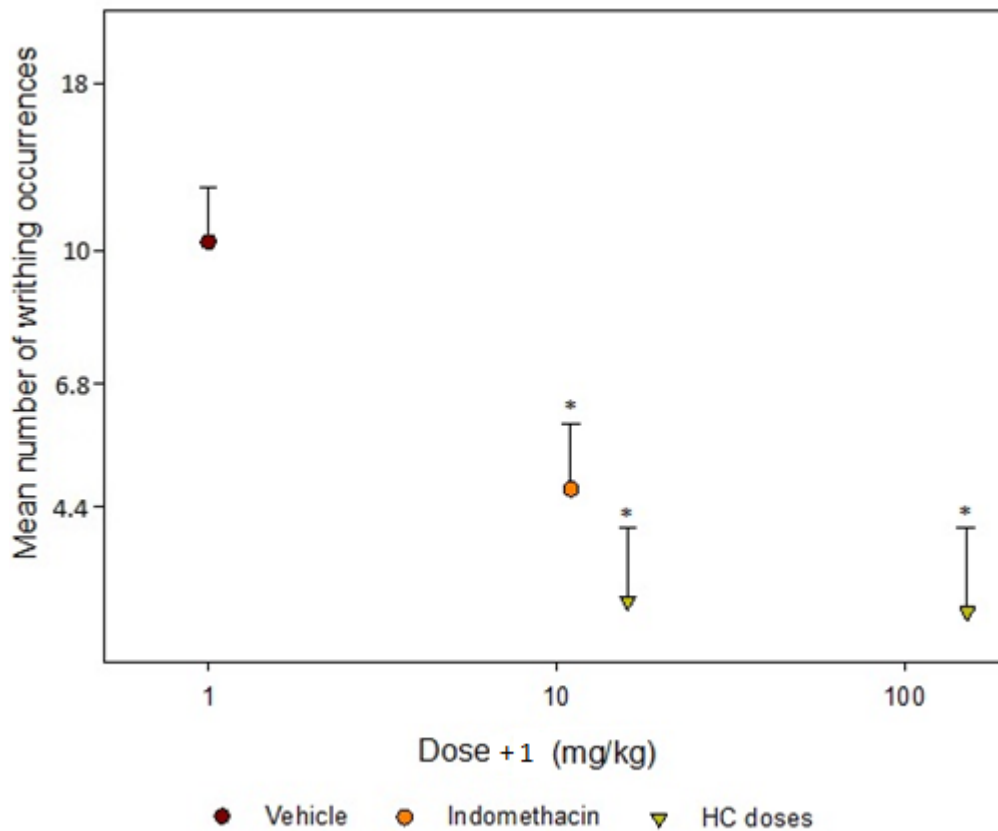


Figure 23 - Number of writhing occurrences in the acetic acid test, evaluation of HC doses and indomethacin. Mean + S.E.M. data for number of writhing occurrences during 20 min in the acetic acid test, for animals treated with vehicle (p.o.), indomethacin 10 mg/kg (p.o.), *H. crenata* (HC) decoction extract 15 mg/kg (p.o.) and 150 mg/kg (p.o.). *Multiple comparisons versus control group (Holm-Sidak method): Overall significance level < 0.05. All data points are n=8 except indomethacin (n=5). (Back-transformed averages of Log10-transformed data, plotted in logarithm scale)

Treatment comparison	Diff of Means	t	Unadjusted P	Critical Level
Vehicle vs. HC 150 mg/kg	0.536	3.802	<0.001	0.017
Vehicle vs. HC 15 mg/kg	0.52	3.686	0.001	0.025
Vehicle vs. Indomethacin	0.359	2.231	0.035	0.05

Table 16 - Multiple comparisons statistic of one way ANOVA for writhing

occurrences. Analyses of writhing observed during 20 min in the acetic acid test for animals treated with *H. crenata* (HC) decoction extract 15 mg/kg (p.o.), 150 mg/kg (p.o.) and indomethacin 10 mg/kg (p.o.) versus control group, vehicle (p.o.). One way ANOVA (Holm-Sidak method), overall significance level <0.05. All data points are n=8 except indomethacin (n=5). Log10 transformed data.

Behaviour	Source of Variation	DF	SS	MS	F	P	Power
Writhing	Between Groups	3	1.488	0.496	6.235	0.003	0.89
	Residual	25	1.989	0.0795			
	Total	28	3.477				

Table 17 - Output of one way ANOVA for writhing occurrences. Analyses of writhing observed during 20 min in the acetic acid test for animals treated with *H. crenata* (HC) decoction extract 15 mg/kg (p.o.), HC 150 mg/kg (p.o.) and indomethacin 10 mg/kg (p.o.) compared with vehicle (p.o.). All data points are n=8 except indomethacin (n=5). (Log10 transformed data)

Treatment comparison	Diff of Means	t	Unadjusted P	Critical Level
Indomethacin vs. HC 150 mg/kg	0.177	1.104	0.28	0.017
Indomethacin vs. HC 15 mg/kg	0.161	1.002	0.326	0.025

Table 18 - Pairwise multiple comparisons statistic of one way ANOVA for writhing

occurrences. Analyses of writhing observed during 20 min in the acetic acid test for animal groups treated with *H. crenata* (HC) decoction extract 15 mg/kg (p.o.), 150 mg/kg (p.o.) and indomethacin 10 mg/kg (p.o.) (Holm-Sidak method). Overall significance level = 0.05. All data points are n=8 except indomethacin (n=5). (Log10 transformed data)

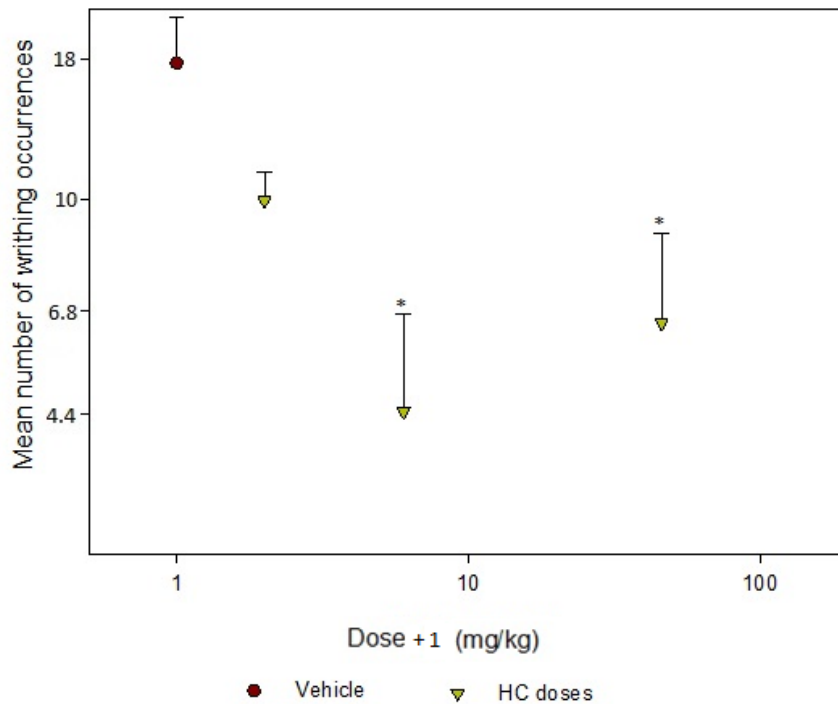


Figure 24 - Number of writhing occurrences in the acetic acid test, evaluation of HC doses response. Mean + S.E.M. data for number of writhing occurrences during 20 min in the acetic acid test, for animals treated with vehicle (p.o.), *H. crenata* (HC) decoction extract 1 mg/kg (p.o.), 5 mg/kg (p.o.) and 45 mg/kg (p.o.). *Multiple comparisons versus control group (Holm-Sidak method): Overall significance level < 0.05. All data points are n=8. (Back-transformed averages of Log10-transformed data, plotted in logarithm scale).

Treatment comparison	Diff of Means	t	Unadjusted P	Critical Level
Vehicle vs. HC 1 mg/kg	0.238	1.359	0.185	0.05
Vehicle vs. HC 5 mg/kg	0.604	3.442	0.002	0.017
Vehicle vs. HC 45 mg/kg	0.45	2.568	0.016	0.025

Table 19 - Multiple comparisons statistic of one way ANOVA for writhing

occurrences. Analyses of writhing observed during 20 min in the acetic acid test for animals treated with *H. crenata* (HC) decoction extract 1 mg/kg (p.o.), 5 mg/kg (p.o.), 45 mg/kg (p.o.) versus control group vehicle (p.o.). One way ANOVA (Holm-Sidak method), overall significance level = 0.05. All data points are n=8. (Log10 transformed data)

Behaviour	Source of Variation	DF	SS	MS	F	P	Power
Writhing	Between Groups	3	1.651	0.55	4.475	0.011	0.716
	Residual	28	3.444	0.123			
	Total	31	5.095				

Table 20 - Output of one way ANOVA for writhing occurrences. Analyses of writhing observed during 20 min in the acetic acid test for animals treated with *H. crenata* (HC) decoction extract 1 mg/kg (p.o.), HC 5 mg/kg (p.o.) and HC 45 mg/kg (p.o.) compared with vehicle (p.o.). All data points are n=8 except vehicle (n=16). (Log10 transformed data).

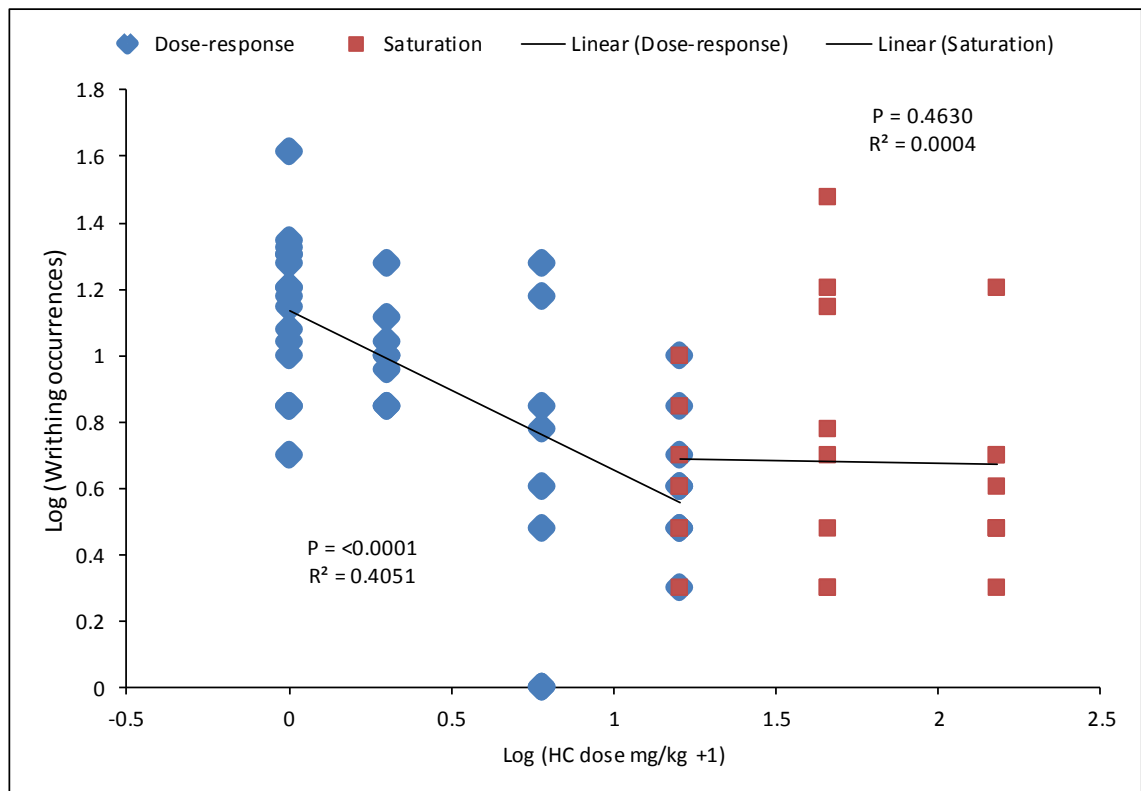


Figure 25 – Dose response of HC doses in the acetic acid test. Data of number of writhing occurrences for each animal during 20 min in the acetic acid test, showing dose response for animals treated with vehicle (p.o.), *H. crenata* (HC) decoction extract 1 mg/kg (p.o.), 5 mg/kg (p.o.), 15 mg/kg (p.o.) and saturation from the doses 15 mg/kg (p.o.), 45 mg/kg and 150 mg/kg (p.o.). All data points are n=8 except vehicle (n=16). The values show the correlation statistics R² and significance values for the two phases of the dose-response relationship.

4.5.2 *Effect of Hyptis crenata decoction fractions and comparison between all treatments tested.*

This study also evaluated the antinociceptive effect of fractions from HC decoction extract (45 mg). These treatments were HC hexane fraction 2.1 mg/kg (p.o.), HC DCM fraction 4 mg/kg (p.o.), HC methanol fraction 14.8 mg/kg (p.o.) and HC water fraction 23 mg/kg (p.o.). Statistical analysis from the results of these evaluations showed that only the hexane fraction was able to reduce writhing during the acetic acid test (Figure 26, Table 21 and Table 22). In order to analyse if there was a difference between the effect of the different fractions and the HC decoction, all the data from both experiments were plotted, and the HC doses linear regression line was used to compare the effect between the treatments. The regression line showed that the hexane fraction was more efficient than indomethacin, and that the effect of HC decoction extract was the same as that of indomethacin (Table 25). However, pairwise comparisons (Table 23) showed that the effect of the fractions and its whole extract (HC 45 mg/kg (p.o.)) were not different from each other regarding the reduction in writhing. But considering that hexane was less concentrated it had a greater antinociceptive effect per mg comparing with the unfractionated extract, while this was not the case for any of the other fractions nor for indomethacin. In addition, a one way ANOVA was carried out with all HC doses and fractions compared to vehicle confirming the significant effect for the hexane fraction, HC 5 mg/kg (p.o.), HC 15 mg/kg (p.o.) and HC 150 mg/kg (p.o.), but HC 45 mg/kg (p.o.) was not significantly different from the control in this analysis (Table 24 and Table 25).

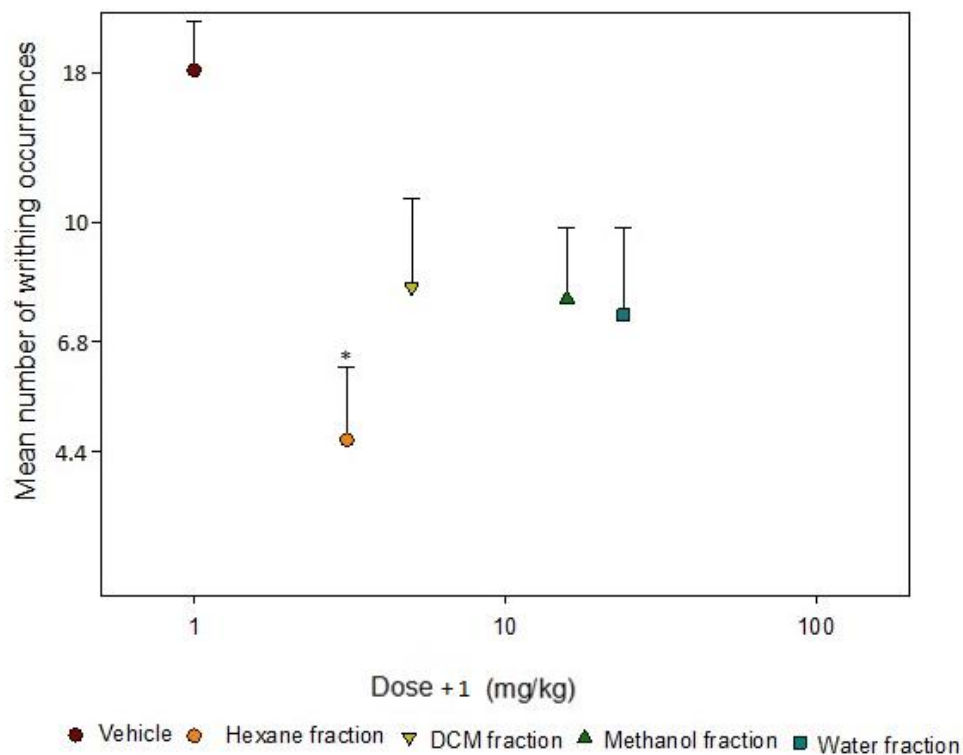


Figure 26 - Number of writhing occurrences in the acetic acid test, evaluation of HC decoction extract fractions. Mean + S.E.M. data for number of writhing occurrences during 20 min in the acetic acid test, for animals treated with (p.o.), *H. crenata* (HC) decoction extract fractions (hexane 2.1 mg/kg (p.o.), DCM 4 mg/kg (p.o.), methanol 14.8 mg/kg (p.o.) and water 23 mg/kg (p.o.)). *Multiple comparisons versus control group (Holm-Sidak method): Overall significance level < 0.05. All data points are n=8. (Back-transformed averages of Log10-transformed data, plotted in logarithm scale).

Treatment comparison	Diff of Means	t	Unadjusted P	Critical Level
Vehicle vs. hexane fraction	0.724	3.454	0.001	0.013
Vehicle vs. water fraction	0.481	2.298	0.028	0.017
Vehicle vs. methanol fraction	0.422	2.012	0.052	0.025
Vehicle vs. DCM fraction	0.412	1.968	0.057	0.05

Table 21 - Multiple comparisons statistic of one way ANOVA for writhing occurrences. Analyses of writhing observed during 20 min in the acetic acid test for animals treated with *H. crenata* (HC) decoction extract fractions (hexane 2.1 mg/kg (p.o.), DCM 4 mg/kg (p.o.), methanol 14.8 mg (p.o.) and water 23 mg/kg (p.o.)) compared with vehicle (p.o.), (Log10 data). All data points are n=8.

Behaviour	Source of Variation	DF	SS	MS	F	P	Power
Writhing	Between Groups	4	2.174	0.544	3.095	0.028	0.557
	Residual	35	6.147	0.176			
	Total	39	8.322				

Table 22 - Output of one way ANOVA for writhing occurrences. Analyses of writhing observed during 20 minutes in the acetic acid test for animals treated with *H. crenata* (HC) decoction extract fractions (hexane 2.1 mg/kg (p.o.), DCM 4 mg/kg (p.o.), methanol 14.8 mg (p.o.) and water 23 mg/kg (p.o.)). All data points are n=8. (Log10 transformed data)

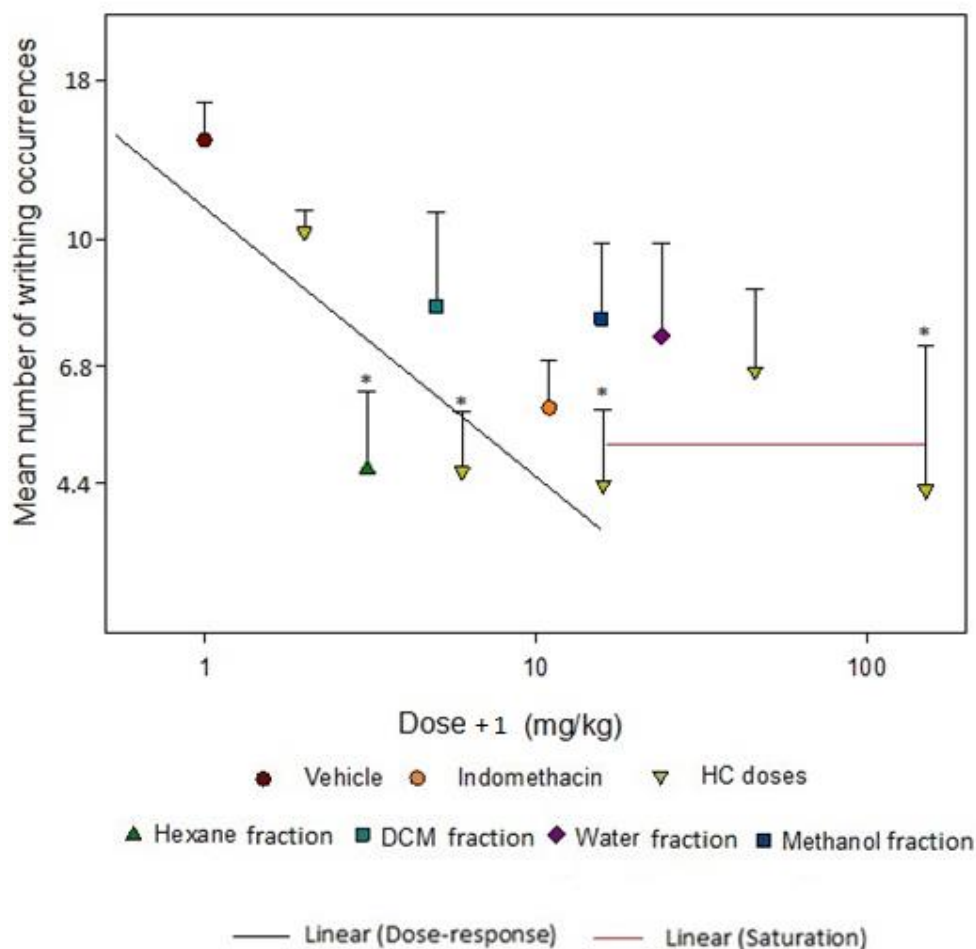


Figure 27 – Dose response of HC doses and decoction fractions. Mean + S.E.M. data with linear regression for the number of writhing occurrences observed during 20 minutes in the acetic acid test for animals treated with *H. crenata* (HC) decoction extract 1 mg/kg (p.o.), 5 mg/kg (p.o.), 15 mg/kg (p.o.), 45 mg/kg (p.o.) and 150 mg/kg (p.o.) and its fractions (hexane fraction 2.1 mg/kg, (p.o.), DCM fraction 4 mg/kg (p.o.), methanol fraction 14.8 mg/kg (p.o.), water fraction 23 mg/kg (p.o.)), indomethacin 10 mg/kg (p.o.) and vehicle (p.o.). This graph uses the dose response curve previously presented in Figure 25 and the indomethacin was plotted as an additional data point in the same coordinates for comparison with *H. crenata* full extract. *Multiple comparisons versus vehicle group (Holm-Sidak method): Overall significance level < 0.05. All data points are n=8 except indomethacin (n=5) and vehicle (n=16). (Back-transformed averages of Log₁₀-transformed data, plotted in logarithm scale).

Behaviour	Source of Variation	DF	SS	MS	F	P	Power
Writhing	Between Groups	4	0.318	0.0796	0.539	0.708	0.05
	Residual	35	5.173	0.148			
	Total	39	5.492				

Table 23 - Output of one way ANOVA for comparison of writhing occurrences.

Analyses of writhing observed during 20 min in the acetic acid test for animals treated with *H. crenata* (HC) decoction extract 45 mg/kg and its fractions (hexane fraction 2.1 mg/kg (p.o.), DCM fraction 4 mg/kg (p.o.), methanol fraction 14.8 mg/kg (p.o.) and water fraction 23 mg/kg (p.o.)). All data points are n=8. (Log10 transformed data)

Treatment comparisons	Diff of Means	T	Unadjusted P	Critical Level
Vehicle vs. Indomethacin	0.408	2.448	0.017	0.01
Vehicle vs. HC 1 mg/kg	0.14	0.995	0.322	0.05
Vehicle vs. HC 5 mg/kg	0.505	3.59	<0.001	0.006
Vehicle vs. HC 15 mg/kg	0.524	3.727	<0.001	0.006
Vehicle vs. HC 45 mg/kg	0.352	2.501	0.014	0.009
Vehicle vs. HC 150 mg/kg	0.533	3.786	<0.001	0.005
Vehicle vs. hexane fraction	0.501	3.563	<0.001	0.007
Vehicle vs. DCM fraction	0.254	1.804	0.075	0.025
Vehicle vs. methanol fraction	0.272	1.932	0.057	0.017
Vehicle vs. water fraction	0.299	2.126	0.036	0.013

Table 24 - Multiple comparisons statistic of one way ANOVA for writhing

occurrences. Analyses of writhing observed during 20 min in the acetic acid test for animals treated with *H. crenata* (HC) decoction extract 1 mg/kg (p.o.), 5 mg/kg (p.o.), 15 mg/kg (p.o.), 45 mg/kg (p.o.) and 150 mg/kg (p.o.) its fractions (hexane fraction 2.1 mg/kg (p.o.), DCM fraction 4 mg/kg (p.o.), methanol fraction 14.8 mg/kg (p.o.) and water fraction 23 mg/kg (p.o.)) and indomethacin 10 mg/kg (p.o.) compared with vehicle (p.o.). All data points are n=8 except indomethacin (n=5) and vehicle (n=16). (Log10 transformed data).

Behaviour	Source of Variation	DF	SS	MS	F	P	Power
Writhing	Between Groups	10	3.231	0.323	3.058	0.002	0.862
	Residual	82	8.662	0.106			
	Total	92	11.892				

Table 25 - Output of one way ANOVA for writhing occurrences. Analyses of writhing observed during 20 min in the acetic acid test for animals treated with *H. crenata* (HC) decoction extract 1 mg/kg (p.o.), 5 mg/kg (p.o.), 15 mg/kg (p.o.), 45 mg/kg (p.o.) and 150 mg/kg (p.o.) its fractions (hexane fraction 2.1 mg/kg (p.o.), DCM fraction 4 mg/kg (p.o.), methanol fraction 14.8 mg/kg (p.o.), water fraction 23 mg/kg (p.o.)) and indomethacin 10 mg/kg (p.o.) compared with vehicle. All data points are n=8 except indomethacin (n=5) and vehicle (n=16). (Log10 transformed data)

4.5.3 Effect of *Hyptis crenata* and its fractions on other behaviour during acetic acid test

During the acetic acid test, in addition to writhing, total time stationary (TSAC), twitching, exploratory and locomotor activities (ELA) were analysed. The one way ANOVA for these behaviours showed no significant effects on TSAC (Table 26 and Table 27) or ELA (Table 28 and Table 29) when compared to vehicle for *H. crenata* extracts doses 1 mg/kg (p.o.), 5 mg/kg (p.o.), 15 mg/kg (p.o.), 45 mg/kg (p.o.), 150 mg/kg (p.o.), HC fractions (hexane fraction, DCM fraction, methanol fraction and water fraction) and indomethacin 10 mg/kg (p.o.) during the acetic acid test. Also no significant effect of these treatments was observed for twitching when compared to the vehicle (Figure 28). But there was a statistically significant difference observed between the groups (Table 30 and Table 31), it was due to the higher number of twitching occurrences from HC 45 mg/kg and DCM fraction groups. However the fact that twitching was not normally distributed even after log transformation makes this difference between treatments doubtful.

Behaviour	Treatments	N	Mean	S.E.M.
TSAC (seconds)	Vehicle	16	322.8	31.9
	Indomethacin	5	266.2	83.7
	HC 1 mg/kg	8	367.6	44.6
	HC 5 mg/kg	8	395.6	33.4
	HC 15 mg/kg	8	217.8	54.8
	HC 45 mg/kg	8	439.1	45.8
	HC 150 mg/kg	8	298.8	55.0
	Hexane fraction	8	297.2	61.9
	DCM fraction	8	337.1	62.6
	Methanol fraction	8	394.1	44.1
	Water fraction	8	341.4	26.3

Table 26 - Measurement of total time stationary during the acetic acid test (TSAC).

Mean and S.E.M. data for TSAC for animals treated with vehicle (p.o.), indomethacin 10 mg/kg (p.o.), HC 1 mg/kg (p.o.), HC 5 mg/kg (p.o.), HC 15 mg/kg (p.o.), HC 45 mg/kg (p.o.), HC 150 mg/kg (p.o.) and HC fractions: hexane fraction (p.o.), DCM fraction (p.o.), methanol fraction (p.o.) and water fraction (p.o.). All data points are n=8 except indomethacin (n=5) and vehicle (n=16). (Back-transformed averages of the differences between Log10 transformed values before and after treatment)

Behaviour	Source of Variation	DF	SS	MS	F	P	Power
TSAC	Between Groups	10	1.163	0.116	1.559	0.134	0.266
	Residual	82	6.118	0.0746			
	Total	92	7.281				

Table 27 - Output of one way ANOVA for total time stationary during the acetic acid test. Effect of animals treated with vehicle (p.o.), indomethacin 10 mg/kg (p.o.), HC 1 mg/kg (p.o.), HC 5 mg/kg (p.o.), HC 15 mg/kg (p.o.), HC 45 mg/kg (p.o.), HC 150 mg/kg (p.o.) and HC hexane fraction (p.o.), DCM fraction (p.o.), methanol fraction (p.o.) and water fraction (p.o.) on TSAC (total time stationary during acetic acid test). All data points are n=8 except indomethacin (n=5) and vehicle (n=16). (Differences between Log10 transformed values before and after treatment).

Behaviour	Treatments	N	Mean	S.E.M.
Total ELA	Vehicle	8	46.0	9.1
	Indomethacin	8	73.3	16.3
	HC 1 mg/kg	8	54.1	6.3
	HC 5 mg/kg	8	37.8	7.3
	HC 15 mg/kg	8	58.1	9.2
	HC 45 mg/kg	8	44.9	10.1
	HC 150 mg/kg	8	61.7	10.0
	Hexane fraction	8	46.7	17.6
	DCM fraction	8	48.9	5.6
	Methanol fraction	8	48.5	9.3
Water fraction	8	50.0	6.3	

Table 28 - Total number of occurrences for exploratory and locomotor activities (ELA). Mean and S.E.M. data for (ELA) for animals treated with vehicle (p.o.), indomethacin 10 mg/kg (p.o.), HC 1 mg/kg (p.o.), HC 5 mg/kg (p.o.), HC 15 mg/kg (p.o.), HC 45 mg/kg (p.o.), HC 150 mg/kg (p.o.) and HC hexane fraction (p.o.), DCM fraction (p.o.), methanol fraction (p.o.) and water fraction (p.o.) during the acetic acid test. All data points are n=8 except indomethacin (n=5) and vehicle (n=16). (Back-transformed averages of the differences between Log10 transformed values before and after treatment).

Behaviour	Source of Variation	DF	SS	MS	F	P	Power
ELA	Between Groups	10	0.413	0.0413	0.727	0.697	Power
	Residual	82	4.662	0.0569			0.05
	Total	92	5.076				

Table 29 - Output of one way ANOVA for total number of occurrences for exploratory and locomotor activities (ELA). Effect on animals treated with vehicle (p.o.) indomethacin 10 mg/kg (p.o.), HC 1 mg/kg (p.o.), HC 5 mg/kg (p.o.), HC 15 mg/kg (p.o.), HC 45 mg/kg (p.o.), HC 150 mg/kg (p.o.) and HC hexane fraction (p.o.), DCM fraction (p.o.), methanol fraction (p.o.) and water fraction (p.o.) on total exploratory and locomotor activities (ELA). All data points are n=8 except indomethacin (n=5) and vehicle (n=16). (Differences between Log10 transformed values before and after treatment).

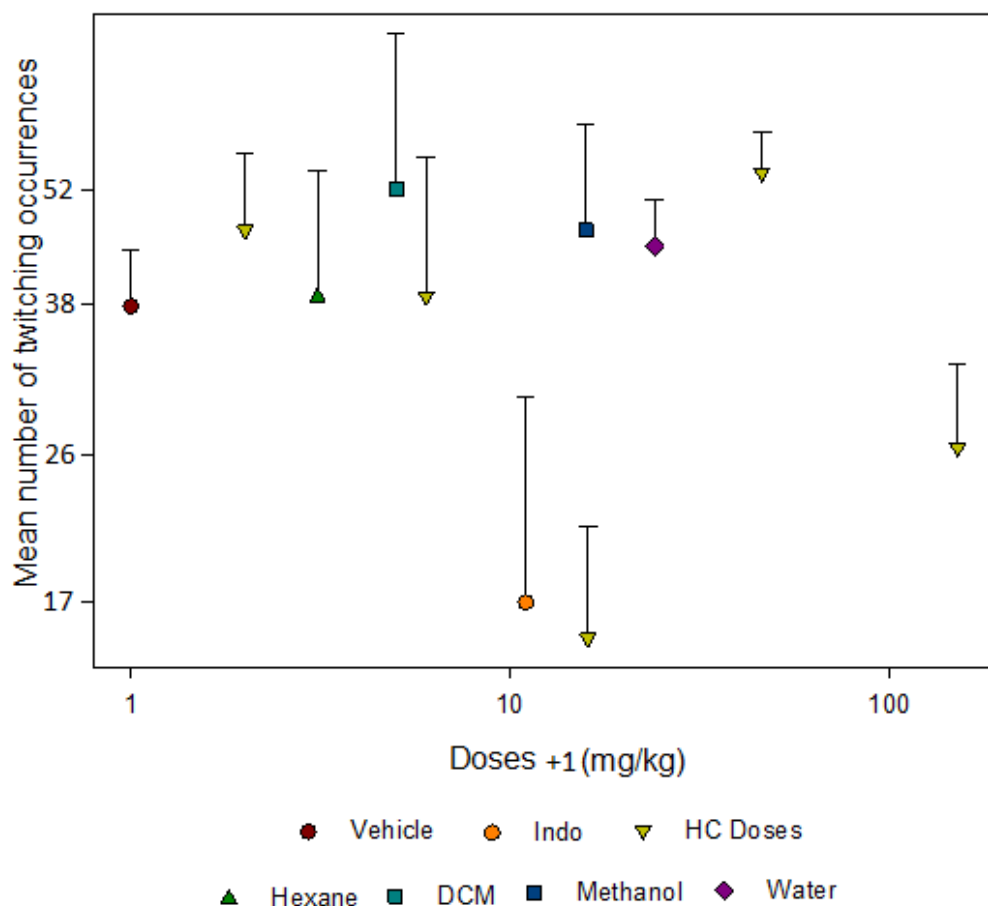


Figure 28 – Number of twitching occurrences in the acetic acid test, evaluation of indomethacin, HC doses and fractions. Mean + S.E.M. data for number of twitching occurrences during 20 min in the acetic acid test, for animals treated with vehicle (p.o.), indomethacin 10 mg/kg (p.o.), HC 1 mg/kg (p.o.), HC 5 mg/kg (p.o.), HC 15 mg/kg (p.o.), HC 45 mg/kg (p.o.), HC 150 mg/kg (p.o.), HC hexane fraction (p.o.), DCM fraction (p.o.), methanol fraction (p.o.) and water fraction (p.o.). All data points are n=8 except indomethacin (n=5) and vehicle (n=16). (Back-transformed averages of the differences between Log10 transformed values before and after treatment, plotted in logarithm scale).

Treatment comparison	Diff of Means	t	Unadjusted P	Critical Level
Vehicle vs. HC 1 mg/kg	0.091	0.593	0.555	0.01
Vehicle vs. HC 5 mg/kg	0.012	0.0782	0.938	0.025
Vehicle vs. HC 15 mg/kg	0.397	2.587	0.011	0.005
Vehicle vs. HC 45 mg/kg	0.159	1.038	0.302	0.007
Vehicle vs. HC 150 mg/kg	0.169	1.102	0.274	0.006
Vehicle vs. Hexane	0.0109	0.0713	0.943	0.05
Vehicle vs. DCM	0.14	0.914	0.363	0.009
Vehicle vs. Methanol	0.0903	0.588	0.558	0.013
Vehicle vs. Water	0.0719	0.468	0.641	0.017
Vehicle vs. Indomethacin	0.354	1.949	0.055	0.006

Table 30 - Multiple comparisons statistic of one way ANOVA for twitching

occurrences. Analysis of twitching observed during 20 min in the acetic acid test for animals treated with *H. crenata* (HC) decoction extract 1 mg/kg (p.o.), 5 mg/kg (p.o.), 15 mg/kg (p.o.), 45 mg/kg (p.o.) and 150 mg/kg (p.o.) its fractions (hexane fraction 2.1 mg/kg (p.o.), DCM fraction 4 mg/kg (p.o.), methanol fraction 14.8 mg/kg (p.o.) and water fraction 23 mg/kg (p.o.)) and indomethacin 10 mg/kg (p.o.) compared with vehicle (p.o.). All data points are n=8 except indomethacin (n=5) and vehicle (n=16). (Log10 transformed data).

Source of Variation	DF	SS	MS	F	P	Power
Between Groups	10	2.624	0.262	2.086	0.035	0.526
Residual	82	10.314	0.126			
Total	92	12.938				

Table 31 - Output of one way ANOVA for twitching. Analysis of effect on animals treated with vehicle (p.o.), indomethacin 10 mg/kg (p.o.), HC 1 mg/kg (p.o.), HC 5 mg/kg (p.o.), HC 15 mg/kg (p.o.), HC 45 mg/kg (p.o.), HC 150 mg/kg (p.o.) and HC hexane fraction (p.o.), DCM fraction (p.o.), methanol fraction (p.o.) and water fraction (p.o.) on twitching. All data points are n=8 except indomethacin (n=5) and vehicle (n=16). (Differences between Log10 transformed values before and after treatment)

4.5.4 Effect of *Hyptis crenata* extract on basal behaviour

The next stage of this study was to investigate whether *H. crenata* decoction and its fractions were having effects on exploratory and locomotor behaviour of mice. In order

to examine this possibility, analyses of behaviour were carried out before and after the administration of HC doses 1 mg/kg (p.o.), 5 mg/kg (p.o.) and 45 mg/kg (p.o.) and the fractions. These behaviours were also analysed during the acetic acid test.

It was observed that the mice displayed the same type of behaviour before and after treatment with HC doses and fractions, these behaviours were: drinking, eating, sniffing, digging, grooming, climbing, hanging, rearing-up, walking and time stationary, the description of these behaviours are presented in the methodology section. From these analyses it was also possible to confirm that the animals did not display writhing and twitching as was observed during the acetic acid test. In addition, from these analyses it was observed that the stationary time was substantially increased due to the effect of acetic acid injection, the average of total stationary behaviour before the treatments and the acetic acid test was 13 seconds whereas during the acetic acid test it was 369 seconds. The paired t-test comparing all groups before the treatments and after the acetic acid injection is shown in Table 32.

In order to analyse the effect of HC decoction doses and its fraction on ethological behaviours observed, they were categorised according to their potential indicators of effect. These effects were sedation (BIS), locomotor impairment and neuroleptic effect (BLY) and total time stationary and hanging as indicators of locomotor activity and muscle relaxation.

The one way ANOVA analyses of differences in behaviours before and after treatments showed that the *H. crenata* 1 mg/kg (p.o.), 5 mg/kg (p.o.), 15 mg/kg (p.o.), 45 mg/kg (p.o.) and 150 mg/kg (p.o.) and its fractions did not induce a significant change for any of the behaviour categories analysed (BIS, BLY, total time stationary and hanging), as shown in Table 33, Table 34, Table 35 and Table 36.

Stationary time	N	Mean (seconds)	Std Dev	SEM	P	t	DF	Power
Before treatments	64	13.176	8.628	1.078	<0.001	22.408	63	1
After injection	64	369.512	128.694	16.087				
Difference	64	356.337	127.217	15.902				

Table 32 – Output of paired-t-test of the change in stationary time that occurred between baseline and after acetic acid injection. The analysis was performed with all groups in which behaviour analyses before and after acetic acid injection was performed.

Behaviour	Treatment	N	Mean	S.E.M.
BIS (Log10 back - transformed data)	Vehicle	8	1.00	0.27
	HC 1 mg/kg	8	2.20	1.14
	HC 5 mg/kg	8	1.18	0.83
	HC 45 mg/kg	8	1.18	0.19
BLY not log transformed	Vehicle	8	-12.00	9.75
	HC 1 mg/kg	8	-2.88	17.10
	HC 5 mg/kg	8	-40.75	11.89
	HC 45 mg/kg	8	-26.63	11.19
Stationary latency (seconds) (Log10 back - transformed data)	Vehicle	8	3.10	1.02
	HC 1 mg/kg	8	2.36	1.46
	HC 5 mg/kg	8	1.51	1.26
	HC 45 mg/kg	8	2.49	2.08
Hanging (seconds) (Log10 back - transformed data)	Vehicle	8	2.90	1.71
	HC 1 mg/kg	8	4.37	3.88
	HC 5 mg/kg	8	1.42	1.82
	HC 45 mg/kg	8	1.38	1.05

Table 33 – Effect of HC treatments on different baseline behaviours. Mean and standard error of mean (S.E.M.) of differences between values before and after treatment for categorized behaviours BIS (sum of sniffing, grooming and rearing), BLY (sum of digging, climbing and walking), behaviours hanging latency and total time stationary, for animal groups treated with vehicle (p.o.), *H. crenata* (HC) decoction extract 1 mg/kg (p.o.), 5 mg/kg (p.o.), and 45 mg/kg (p.o.). Some data are back-transformed averages of the differences between Log10 transformed values before and after treatment).

Behaviours	Source of Variation	DF	SS	MS	F	P	Power
BIS	Between Groups	3	0.549	0.183	0.904	0.451	0.049
	Residual	28	5.663	0.202			
	Total	31	6.212				
BLY	Between Groups	3	6643.625	2214.542	1.693	0.191	0.173
	Residual	28	36616.25	1307.723			
	Total	31	43259.88				
Stationary (latency seconds)	Between Groups	3	0.413	0.138	0.347	0.791	0.049
	Residual	28	11.102	0.397			
	Total	31	11.515				
Hanging	Between Groups	3	1.438	0.479	0.784	0.513	0.049
	Residual	28	17.12	0.611			
	Total	31	18.559				

Table 34 - Output of one way ANOVA for effect of HC treatments on different baseline behaviours. Effect of animal groups treated with vehicle (p.o.) *H. crenata* (HC) decoction extract 1 mg/kg (p.o.), 5 mg/kg (p.o.) and 45 mg/kg (p.o.) on the categorized behaviours; BIS (sum of sniffing, grooming and rearing), BLY (sum of digging, climbing and walking), hanging latency and total time stationary. All data points are n=8. (Some data are differences between log₁₀ transformed values before and after treatment).

Behaviour	Treatment	N	Mean	S.E.M.
BIS (Log 10 transformed)	Vehicle	8	1.00	4.21
	Hexane fraction	8	9.00	2.82
	DCM fraction	8	7.75	5.52
	Methanol fraction	8	0.75	5.86
	Water fraction	8	4.00	8.17
BLY (not log transformed)	Vehicle	8	-12.00	9.75
	Hexane fraction	8	-5.75	15.55
	DCM fraction	8	12.00	13.92
	Methanol fraction	8	-1.38	10.65
	Water fraction	8	-9.63	15.31
Stationary latency (seconds) (Log 10 transformed)	Vehicle	8	3.10	1.02
	Hexane fraction	8	2.19	1.18
	DCM fraction	8	1.41	1.62
	Methanol fraction	8	1.15	0.37
	Water fraction	8	2.66	0.68
Hanging (seconds) (Log 10 transformed)	Vehicle	8	2.90	1.71
	Hexane fraction	8	1.38	1.30
	DCM fraction	8	1.38	1.55
	Methanol fraction	8	1.29	1.06
	Water fraction	8	1.87	2.29

Table 35 - Effect of HC decoction fractions treatments on different baseline behaviours. Mean and standard error of mean (S.E.M.) for categorized behaviours; BIS (sum of sniffing, grooming and rearing), BLY (sum of digging, climbing and walking), hanging latency and total time stationary, for animal groups treated with vehicle (p.o.), HC hexane fraction 2.1 mg/kg (p.o.), HC DCM fraction 4 mg/kg (p.o.), HC methanol fraction 14.8 mg/kg (p.o.). All data points are n=8. (Back-transformed averages of the differences between Log10 transformed values before and after treatment).

Behaviours	Source of Variation	DF	SS	MS	F	P	Power
BIS (Log 10 transformed)	Between Groups	4	459	114.75	0.457	0.767	0.049
	Residual	35	8795	251.286			
	Total	39	9254				
BLY (not log transformed)	Between Groups	4	2875.85	718.962	0.512	0.728	0.05
	Residual	35	49179.25	1405.121			
	Total	39	52055.1				
Stationary (latency seconds) (Log 10 transformed)	Between Groups	4	1.07	0.268	0.903	0.473	0.473
	Residual	35	10.369	0.296			
	Total	39	11.439				
Hanging (seconds) (Log 10 transformed)	Between Groups	4	0.686	0.172	0.256	0.904	0.904
	Residual	35	23.438	0.67			
	Total	39	24.124				

Table 36 - Effect of HC decoction fractions treatments on different baseline behaviours. Output of one way ANOVA for effect of animal groups treated with vehicle (p.o.), HC hexane fraction 2.1 mg/kg (p.o.), HC DCM fraction 4 mg/kg (p.o.), HC methanol fraction 14.8 mg/kg (p.o.) and HC water fraction 23 mg/kg (p.o.) on the categorised behaviours; BIS (sum of sniffing, grooming and rearing), BLY (sum of digging, climbing and walking), hanging latency and total time stationary. All data points are n=8. (Differences between Log10 transformed values before and after treatment).

4.6 Discussion

The main aim of the experiments described in this chapter was to evaluate the effect of *H. crenata* decoction and its fraction on reducing writhing in an animal model of visceral pain. The results of these experiments showed that *H. crenata* decoction extract at a dose of 15 mg/kg (p.o.) and 150 mg/kg (p.o.) significantly decreased the number of writhings, suggesting a possible effect of *H. crenata* compounds on the visceral nociceptive system. As expected, this effect was also observed for the treatment with indomethacin 10 mg/kg (p.o.), this drug is known for its ability to decrease prostaglandin levels and researchers have reported that this pain mediator increases its

amount in the peritoneal fluid after injection of acetic acid (Collier *et al.*, 1968). This fact correspondingly could indicate that *H. crenata* decoction compounds could be also decreasing prostaglandin levels.

Although the acetic acid test has the benefit of being very sensitive to detect even weak analgesic effects, it has the disadvantage of having a low specificity, which means that substances that are adrenergic blockers, antihistamines, muscle relaxants, monoamine oxidase inhibitors and neuroleptics could have a positive effect in the test (Le Bars *et al.*, 2001). It is possible that any of these mechanisms could be responsible for the antinociceptive effect observed in the acetic acid test and also in the thermal nociception test (Hargreaves test). Adrenergic blockers, antihistamines and monoamine oxidase inhibitors have also been reported to induce or enhance analgesia in the hot plate test. (Bianchi *et al.*, 1992; Rojas-Corrales *et al.*, 2000; Galeotti *et al.*, 2002; Gürel *et al.*, 2009). In addition, a muscle relaxant effect has been reported to be a false positive for this test. Considering that *H. crenata* treatments could be acting in any of these potential mechanisms, it was decided that the next step of the experiment was to do a preliminary evaluation of the animal behaviour that would indicate a correlation with these effects. Because the next stage of this study would be to evaluate the dose-dependent response of lower and higher doses than HC 15 mg/kg and the antinociceptive effect of HC decoction fractions, the behaviour analyses was carried out for these treatment groups. Therefore, if *H. crenata* doses or its fractions were having an effect via the antihistaminic pathway, it is very likely that a decrease would be observed in the number of sniffing, grooming and rearing events categorised as an indicator of sedation, since it is well known that antihistaminic agents provoke sedation as an adverse effect (Hindmarch and Shamsi, 1999; Shamsi and Hindmarch, 2000; Bender *et al.*, 2003). However, if *H. crenata* would have neuroleptic-like action similar to apomorphine that is a dopamine receptor agonist, it is likely that the extract would affect the occurrence of digging, climbing and walking behaviour categorised as indicators of neuroleptic effects (Puech *et al.*, 1978; Lassen, 1979; Kendler *et al.*, 1982). But if the antinociceptive effects observed were due to the fact that HC groups had a muscle relaxant effect, these animals would decrease hanging latency and increase total time stationary due to motor impairment. Because none of these behaviours were specifically affected by *H. crenata* decoction and its fractions, it could be suggested that the antinociceptive effect observed in *H. crenata* treatments might not be due to antihistaminic, muscle relaxant, or dopamine agonist action. In addition, the fact that none of the decoction fractions

altered the behaviours analysed reinforces this hypothesis, considering that any potential change of these behaviours analysed would theoretically be more evident since the process of fractionation selectively purifies the compound in the sample, and any effect induced by this compound might be noticed or amplified. Another possibility is that there was involvement of these mechanisms, however due to low statistical power the analyses failed to detect the change on the animal behaviour.

A further objective of this experiment was to investigate the dose response curve of *H. crenata* extract. The analyses showed that 5, 15, 45 and 150 mg/kg were effective; however no effect was observed for 1 mg/kg, suggesting that the antinociceptive effect is induced above of this dose, with a threshold for effect between 1 and 5 mg/kg. The linear regression analyses of the effect of all HC doses tested, indicated that there was dose-response until 15 mg/kg, however this effect reached maximal efficacy between this dose and 45 mg/kg, becoming saturated. As described in the introduction (Section 1.4.3), there are eight previous antinociceptive studies that evaluated the activity of three other *Hyptis* species (Bispo *et al.*, 2001; Lisboa *et al.*, 2006; Marcal *et al.*, 2006; Menezes *et al.*, 2007; Santos *et al.*, 2007b; Arrigoni-Blank *et al.*, 2008; Franco *et al.*, 2011; Raymundo *et al.*, 2011); the acetic acid inducing writhing was used in all these studies. Four of these studies evaluated *Hyptis* essential oil and the others evaluated an extract and fractions. The dose used in these studies ranged from 10 to 400 mg/kg for the essential oils studies, 100 to 400 mg/kg in the aqueous studies and 25 to 400 mg/kg for extractions using organic solvents studies. The essential oils evaluated were from the *H. pectinata* and *H. fruticosa* species, one of these studies reported saturation in the effect of essential oil at 50 mg/kg (i.p.) (Franco *et al.*, 2011), while for the other studies it was not possible to indicate which dose reached the maximum response since the lowest dose used was already effective (Arrigoni-Blank *et al.*, 2008). For the aqueous extract that would be more comparable with this *H. crenata* study, as this experiment also used aqueous extraction, it is not very clear if there was dose-dependency of the observed writhing effect. However, it appears that *H. suaveolens* treatment reached its maximum effect between 200 and 400 mg/kg (p.o.) (Santos *et al.*, 2007b) and *H. pectinata* was still showing an increase in effect at the highest dose tested that was 400 mg/kg (p.o.) (Bispo *et al.*, 2001). If compared, the predicted maximum effect occurred with a dose of *H. crenata* that is between 15 and 150 mg/kg (p.o.), while these other *Hyptis* studies occurred at much higher doses. There are a number of reasons for this including the fact that the active compounds may be more efficacious or occur at

higher concentrations or that *H. crenata* compounds have a lower “*first-pass loss*” when passing through sites like gastrointestinal tissues and the liver than the compounds present in these other species, therefore a smaller amount of treatment is needed to induce the effect (Rowland and Tozer, 2011). However, because in these other reports on *Hyptis* aqueous extracts the dose-dependency effect was not properly calculated, it could be that the effect was already saturated in the lowest dose tested (100 mg/kg) which in any case is still very high when compared to the present *H. crenata* study.

The results of this experiment also showed that the most active fraction from *H. crenata* decoction extract was the hexane fraction, with no significant effect observed for the other fractions. One of the previous *Hyptis* genus antinociceptive studies also investigated the effect using fractions from *H. pectinata*, however the difference was that the organic solvents (MeOH, hexane, chloroform and ethyl acetate) were added directly to the plant powdered leaves (Lisboa *et al.*, 2006) while the present study first produced a decoction which was then fractionated. In contrast with *H. crenata* results, all these fractions from *H. pectinata* were effective in decreasing writhing, indicating the possibility of a more diverse class of compounds being active on antinociception. But this does not mean that this range of diverse compounds were absent in *H. crenata* species, it is possible that the decoction extract did not select these compounds. It could be argued that a study like that of Lisboa *et al.*, 2006 produces fractions that do not reproduce the profile of compounds that would be selected in a traditional use method. Therefore there is a potential risk for some of these compounds to be toxic, assuming that they have never been “clinically tested” by the medicinal plant users (Fabricant *et al.*, 2001). In comparing the percentage inhibition of writhing between the *H. crenata* and *H. pectinata* studies, it appears that *H. crenata* hexane fraction appeared to be more effective in reducing writhing since 2.1 mg/kg of the fraction was able to reduce writhing by 68% compared to control (present study), whereas 100 mg/kg of the hexane, ethyl acetate and chloroform fractions from *H. pectinata* achieved 62%, 67% and 84%, respectively. Another interesting outcome from the present study is that *H. crenata* decoction extract was as effective as indomethacin, although this was not surprising, since similar results were also found using *H. pectinata* species using aqueous extract as treatment (Bispo *et al.*, 2001). However, after HC fractionation the results showed that hexane fraction was more effective than indomethacin.

When comparing the type of behaviour observed before and after the injection of acetic acid, as expected writhing behaviour only appears for mice after they have received the acetic acid injection. However, what was also noted was that all mice after acetic acid injection, independent of treatment received, displayed a slight tremor designated as twitching behaviour. In addition, animals receiving acetic acid displayed a considerably increased time stationary. A literature search was made to examine if other authors have noted or measured these variables during the acetic acid test. Although an increase in time stationary after acetic acid injection has been described by Le Bars *et al.* (2001) there is no mention of twitching during the acetic acid test. The possibilities for these results could be that because twitching is not a major change in behaviour it was not measured in previous studies. Considering that these two behaviours (time stationary and twitching) could measure the level of pain during the acetic acid test, analyses were carried out comparing *H. crenata* decoction doses, its fractions and indomethacin against the control (vehicle). None of these treatments were significant in decreasing twitching or time stationary, even indomethacin. This could suggest that these behaviours are not caused by pain but it could be associated with some stress or anxiety (Morgan *et al.*, 1999).

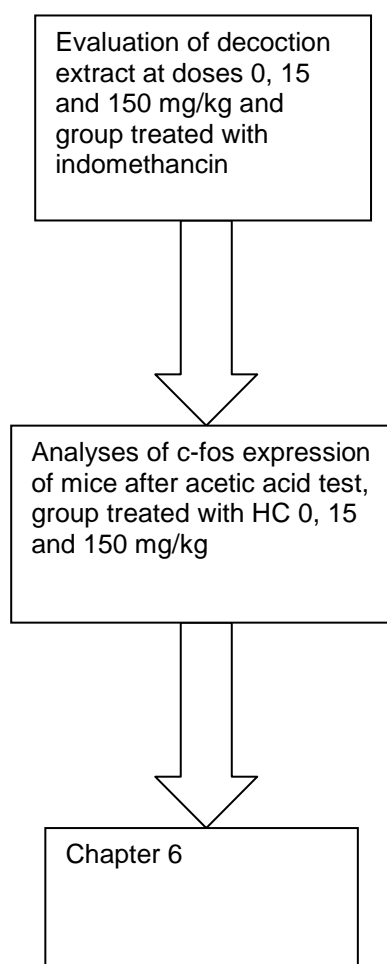
4.7 Conclusions

The data presented in this chapter supports the antinociceptive effect of *H. crenata* decoction extract at doses of 5, 15, 45 and 150 mg/kg (p.o.) and its hexane fraction on visceral pain caused by acetic acid. There is a dose-dependent effect observed for the doses 0, 1, 5 and 15 mg/kg. The saturation in the effect occurred at 15 mg/kg or between 15 and 45 mg/kg (p.o.). Therefore, these results support the antinociceptive effect of dose 15 mg/kg (p.o.) which was derived from reports of traditional use. *H. crenata* decoction extract and its fractions do not affect locomotor and ethological behaviours. Also the twitching and the increase of time stationary induced by acetic acid are not affected by *Hyptis crenata* treatments or by indomethacin.

**Chapter 5. Analysis of Fos-Positive Neurones Expression in Forebrain
and Midbrain of Mice Treated with *Hyptis crenata* after Acetic
Acid Test**

5.1 Overall Procedures

5.1.1 Flowchart



5.2 Introduction

In addition to behavioural indices of antinociceptive action described in the preceding chapters, an aim of these studies was to obtain other evidence that *Hyptis* extract was able to modulate pain pathways. This chapter describes a study in which the expression of the immediate early gene product, Fos, was used to determine neural activation in the brains of animals from the acetic acid test described in Chapter 4.

The use of the protein Fos as a neuron marker for analysing the response to pain stimuli has been used by several research studies. The advantage of this technique is that it is relatively simple to carry out and would give an unbiased measure of the pain response. In addition, the reduction of pain-induced expression of this protein in a specific part of the pain pathway can contribute to the understanding of the pharmacodynamic action and mechanism of a drug. However the disadvantage of this immediate early gene is that it is not only expressed in response to pain stimuli but also to other stimuli like stress (Harris, 1998; Herdegen and Leah, 1998; Hoffman and Lyo, 2002; Sinniger *et al.*, 2004).

The injection of acetic acid used in the acetic acid test as noxious stimulus will induce chemical irritation in the peritoneum area increasing the level of pain mediator substances such as prostaglandin. This has the ability to activate nociceptors, therefore lowering its threshold and transmitting signals to second-order neurones in the spinal cord. From the spinal cord these signals are sent through the anterolateral system towards the thalamus, and from the ventral posterior thalamus the somatosensory information is transmitted to the cortex via third-order neurons. These sensory signals will be processed in the cortex and will generate the perception of pain (Dickenson and Suzuki, 2005; Toates, 2007).

In response to this pain stimulus, the immediate early gene c-fos is expressed and will increase the Fos protein level 40-120 min after acetic acid injection (Harris, 1998). This increase of protein level due to the acetic acid stimulus has been reported to be found in the spinal cord (e.g. superficial laminae of the dorsal horn), forebrain (e.g. hypothalamus), midbrain (e.g. periaqueductal gray matter), as described in Table 37 which shows these different regions with specific parts analysed for c-fos expression after visceral pain induced by acetic acid. Regarding the function for some of these brain areas, the hypothalamus is an area of the brain that is mainly involved in

homeostasis and emotion; this emotion can be related to fear and anxiety. In regard to periaqueductal gray (PAG), it has an important role in the descending control pathway, and reports have suggested that PAG is the main site of action for analgesic drugs like morphine. It has also been reported that inhibitory control from PAG mainly affects transmission of C fibres nociception (Jacquet and Lajtha, 1974; Matthews, 2001; Heinricher *et al.*, 2009).

Authors	Anatomical locations	Methods of visualisation	Animal
(Nakagawa <i>et al.</i> , 2003)	Amygdale central nuclei	C-fos mRNA	Rats
(Bianchi <i>et al.</i> , 2003)	Periaqueductal gray matter (PAG)	Immunohistochemistry	Rats
(Taylor <i>et al.</i> , 1998)	Spinal cord	Immunohistochemistry	Mice
(Huang <i>et al.</i> , 2010)	Spinal cord, superficial dorsal horn	Immuno and western blot	Mice
(Sinniger <i>et al.</i> , 2004)	Paraventricular nucleus of the hypothalamus (PVN)	C-fos mRNA	Rats
(Bonaz <i>et al.</i> , 2000)	Nucleus tractus solitarius, and paraventricular nucleus of the hypothalamus	Immunohistochemistry	Rats
(Snowball <i>et al.</i> , 2000)	Paraventricular nucleus of the hypothalamus (PVN), the supraoptic nucleus (SON), ventral regions of the anterior hypothalamus (PVA) and periaqueductal gray (PAG).	Immunohistochemistry	Rats
(Jiao and Xie, 2004)	Limbic system and anterior cingulate cortex	Immunohistochemistry	Rats
(Mitsui <i>et al.</i> , 2003)	Midbrain periaqueductal gray matter	Immunohistochemistry	Rats
(Hammond <i>et al.</i> , 1992)	Spinal cord and the nucleus tractus solitarii	Immunohistochemistry	Rats
(DeLeo <i>et al.</i> , 1991)	Thoraco-lumbar region of the spinal cord	Immunohistochemistry	Rats

Table 37 - Regions of the central nervous system where c-fos or Fos was observed after intraperitoneal injection of acetic acid

5.2.1 *Fos expression for measurement of antinociceptive properties of medicinal plants and other drugs studies*

Searches were made to find studies that evaluated medicinal plant antinociceptive effects and also used c-fos expression as a measurement tool for pain response. Examining the databases in Medline, Web of Knowledge and Scopus, only one study was found published by Peng *et al.* (2009). This study reported the effects of *Ligustici chuanxiong* Hort essential oil in reducing c-fos expression in cells localised in the brain stem and the hypothalamus of rats after performing the nitroglycerin-induced headache test. However, in relation to conventional analgesic drugs studies and methods of evaluation involving c-fos measurements, there are a number of articles. In these articles drugs have been reported for their ability to suppress c-fos expression in specific areas of the central nervous system using several animal models of pain. These drugs can be divided into two categories; drugs suppressing c-fos at the trigeminal nuclear complex and suppressors of c-fos at the spinal cord and dorsal horn.

5.2.2 *Suppressors of c-fos at the trigeminal nuclear complex*

The trigeminal nerve carries sensory information from the head and the trigeminal nuclear complex has been described to be associated with pain mechanisms involved in primary headaches (Evers, 2010; Fernandez-De-Las-Peas, 2010; Edvinsson, 2011; Hargreaves, 2011; Kelman, 2011). Sumatriptan, a triptan sulfa used for the treatment of migraine, has been reported to reduce c-fos in the trigeminal nucleus caudalis of rats performing a test that consisted of injecting carrageenan into the cisterna magna to induce inflammation (Nozaki *et al.*, 1992). Similarly, the drug dihydroergotamine, an ergot alkaloid also used to treat migraines has been reported to reduce c-fos expression at the superior local superficial laminae of the trigeminal nucleus caudalis (Hoskin *et al.*, 1996), and also in the dorsal horn at levels C1 and C2.

Fentanyl, an agonist at the μ -opioid receptor, also suppresses c-fos in the trigeminal nuclear complex at the transitional region between subnucleus interpolaris and caudalis and within the subnucleus caudalis after noxious stimulation of the dental pulp (Chattipakorn *et al.*, 1999). Finally, celecoxib a sulfa non-steroidal anti-inflammatory drug, selective COX-2 inhibitor and acetaminophen, which is classified as a non-steroidal anti-inflammatory drug, both decreased c-fos expression in the caudalis

subnucleus in a rat model of orthodontic pain involving incisor movement (Stabile *et al.*, 2009).

5.2.3 Suppressors of *c-fos* at the spinal cord

Another drug that has been reported for suppressing *c-fos* is morphine. This drug is a potent opiate analgesic medication and researchers suggest that its effects decrease *c-fos* in the dorsal horn neurons (Burkey *et al.*, 1996). Remifentanyl, which is also an opioid and lidocaine, a common local anesthetic and antiarrhythmic drug, have been reported to inhibit *c-fos* in the spinal cord during the formalin test (Abbadie *et al.*, 1997). Sevoflurane, a halogenated ether, also inhibits *c-fos* cells in the spinal cord after the formalin test (Hao *et al.*, 2002). However, it should not be concluded that the spinal cord is the sole site of action for these drugs, as in most cases only the spinal cord was examined.

5.2.4 Suppressors of *c-fos* at the cingulate cortex

The cingulate cortex has been described to be specially activated by muscle pain, and it was also reported that pain cognition and emotion is segregated in this area (Maeda *et al.*, 2011; Shackman *et al.*, 2011; Takahashi *et al.*, 2011). The drug Milnacipran, which is a serotonin-norepinephrine reuptake inhibitor used in the clinical treatment of fibromyalgia, has the ability to suppress *c-fos* expression in the anterior cingulate cortex (Takeda *et al.*, 2009).

5.3 Aim of this experiment

Evidence shows that the Fos protein can be used as a valid tool for the study of the neural activities correlating with nociception, and research has reported its expression in some areas of the forebrain and midbrain of mice, induced by intraperitoneal injection of acetic acid. Therefore, this experiment aimed to investigate whether *H. crenata* decoction extract, which was effective in reducing acetic acid writhing, would also reduce Fos expression localised in the forebrain and midbrain of the mouse after this noxious stimulus.

5.4 Material and methods

5.4.1 Experimental design

In this experiment the Fos analyses were carried out on mice tissue after performing the acetic acid test (Chapter 4). The forebrain and midbrain were the parts of the central nervous system used for Fos expression analyses. The reason for not analysing the spinal cord was because there was an uncertainty regarding which level of the spinal cord the acetic acid stimulus was acting, therefore it would be difficult to select and analyse precisely the Fos active section and make valid comparisons between treatment groups.

Because the brain is a very large area, it was necessary to decide which specific areas would be the focus of this experiment. A literature search was made to investigate in which areas of the brain the Fos protein is expressed after acetic acid injection in mice. The results of this search showed that the main areas were the forebrain and midbrain, specifically the area of PVA, PVN, SON and PAG (Table 37). Therefore these were the target areas for this study.

The immunohistochemistry technique was the chosen method for Fos protein detection. The reason for choosing immunohistochemistry is due to the fact that it allows using fixed tissue, which is easier to handle for the large number of sections generated. In addition, with this technique it would be possible to integrate the response to a pain stimulus that occurred over a longer period, and it would give a potentially larger signal than the c-fos mRNA transcription technique. Furthermore, this technique has been widely used for studies using Fos as the method for neural marker (Bonaz *et al.*, 2000; Bianchi *et al.*, 2003; Nakagawa *et al.*, 2003).

The details of the animals and the treatments are reported in Section 4.4. This experiment analysed the treatments HC 15 mg/kg (p.o.), HC 150 mg/kg (p.o.) and vehicle, water for injection (p.o.) after the acetic acid test. Note that all animals received the acetic acid injection and the hypothesis being tested was whether *H. crenata* would reduce the level of Fos expression compared with vehicle, there was no comparison made with conventional anaesthetic.

5.4.2 Brain collection and fixation

Ninety min after the injection of acetic acid animals were moved sequentially in their individual cages to a room for fixation and collection of the brain tissue. Animals were not moved until ready for the fixation in order to prevent any additional stimulus.

The mouse was placed in a small plastic chamber (approx. 5 x 5 x 10 cm) containing a tissue on which 1 ml of fluothane had been added. The animal remained in the chamber for approx. 2 min until breathing had ceased and spontaneous recovery could not occur.

The animal was removed and quickly dissected. The pelt over the chest was removed and the rib cage removed by cutting across the line of the diaphragm and, holding the xiphoid process, the anterior chest was dissected. The left jugular was cut and the descending aorta clamped with a small, curved hemostatic clamp.

Gently holding the right ventricle with Watchmakers forceps, approx. 10 ml of heparinised PBS at 4°C was infused into the right ventricle using a 20 ml syringe and 23-gauge needle. The infusion took approx. 1 minute and the paws could be seen to visibly blanch as the blood was removed. The animal was then fixed with 10-15 ml of 4% paraformaldehyde (PFA) at 4°C. Good fixation was indicated by muscular twitching and stiffening of the forelimbs.

After fixation the head was removed, the scalp dissected and the cranial bones peeled off using the hemostatic clamp. The brain was placed in a 5 ml labelled specimen tube containing 4 ml PFA and post fixed at 4°C for 48 h. The PFA was removed and replaced by a solution of 30% sucrose in PBS containing 1 mg/ml sodium azide as an anti-microbial agent and stored at 4°C.

5.4.3 Tissue sectioning

Each brain was sectioned using the cryostat (Microm HM 560). A razor blade was used to make a coronal cut through the cerebellum and the caudal surface of the brain was mounted and fixed on a cryostat chuck using tissue-tek (MICROM international GmbH). The chuck was then fixed to the head stage of the cryostat. The cryostat chamber was maintained at a temperature of -22°C specimen and -20°C knife. The tissue excess was trimmed until the anterior commissure was no longer continuous across the midline.

For collecting the tissue sample the free floating plate technique was used, which consisted of allocating the brain sections into wells of a multiwell plate filled with PBS 0.1M, pH 7.4.

When the anterior commissure was no longer continuous 22 sections were collected (50 µm thick) from the forebrain region. These sections were collected in two sets (11 slices in each well for sample duplication). After the forebrain, 56 sections were cut and discarded until the midbrain was reached. In this area 40 sections were collected (20 slices each well).

During the sectioning, the atlas 'Mouse Brain in Stereotaxic Coordinates' (Paxinos, 1996) was used as reference for the structures. The forebrain sections were collected from interaural line 3.58 mm/bregma -0.22 mm until interaural line 2.10 mm/bregma -1.70 mm and for midbrain collected from interaural line 1.26 mm/bregma 2.54 mm until interaural line 1.40 mm/bregma 5.20 mm.

The tissues samples were transferred from PBS into 5 ml labelled specimen tubes with cryoprotectant (150 ml ethylene glycol, 150 ml glycerol, 100 ml of 0.3 M (PBS) PH 7.2 and 100 ml of deionised water) and stored in a freezer (-24°C).

5.4.4 Immunocytochemistry for visualising Fos protein expression

Immunocytochemistry was carried out on one duplicate of the forebrain sections (11 sections) and midbrain sections (20 sections) for each mouse. These sections were collected from the cryoprotectant liquid and washed with PBS 0.1M three times for 5 min each. Next the sections were permeabilized for 30 min with 0.1% Triton X -100 (Molekuly). After, the sections were placed for overnight incubation with polyclonal rabbit anti-Fos antiserum (Sc-52 Santa Cruz Biotechnology), diluted 1:300 in PBS containing 3% bovine albumin (Sigma Aldrich) and 0.1 M Lysine, (L-2,6 Diaminohexanoic acid, monohydrochlorid FW 182.6) in a large cold room (4°C).

After overnight incubation, the sections were placed for warming back to room temperature (20°C) for 30 min. Then the sections were washed three times for 5 min each. After, they were incubated for 2 h with biotinylated goat anti-rabbit secondary antibody affinity purified (1:100, Vector laboratories, UK).

Sections were washed again with PBS three times (10 min each) followed by 1 h incubation with Fluorescein Avidin D concentration 1:100 (Vector laboratories). During Fluorescein incubation the plate was covered with aluminium foil to protect it from the light. Finally, the sections were washed (three times for 10 min each).

The sections were collected with a fine paint brush from the wells and mounted on to coated microscope slides and left to dry under aluminium foil for 15 min. The sections on the slide were then covered with a fine layer of Vectashield (hard set mounting medium containing the nuclear stain DAPI H-1500 from Vector Laboratories) followed by a cover glass (25x60 mm, 0.13-0.17 mm thick) and left to dry for 15 min. The mounted slides were then stored in plastic slide storage boxes in a fridge (4°).

All washing was performed using a polystyrene plate with wells that were filled with PBS 0.1M (500 µl/well) using a glass Pasteur's pipette whose sucking edge was narrowed with a Bunsen burner to avoid damaging the tissues. After filling with PBS the plate was covered with its lid and placed in a 3D Rocking Platform STRA (Stuart Scientific) at 30 revolutions/min. The brain slices were washed having a maximum of 3 sections in each well.

All the incubation procedures were performed with the plate placed on a 3D Rocking Platform STRA (Stuart Scientific) 30 revolutions/min.

5.4.5 Image processing, visualization Fos detection and counting process

For detecting and counting the number of Fos- positive neurones, a Leica DMRA fluorescence microscope model C4742-80-12AG was used, using filter set Cy7 and a digital image processing software for microscope, Axiovision 4.8.

Firstly, the sections were observed in the microscope to detect where Fos was expressed. In the forebrain Fos protein expression was detected in the PVA, PVN and SON area. In the midbrain Fos was also detected in the PAG, but due to the inconsistent and small signal it was not counted. The PVA and SON were counted using 20x magnification and PVN using 10x magnification. In order to have the same size area counted, each structure was measured as shown in Figure 29 (PVA: 0.35 mm x 0.2 mm (A), PVN: 0.75 mm x 0.25 mm for each side (B) and SON: length of area 0.3 mm and 0.01, 0.04 and 0.02 widths).

All sections with Fos expression in the PVA, PVN and SON areas were counted. There were no more than four sections for each mouse containing Fos expression in the PVA and PVN, and no more than two sections for SON. The statistical analyses were carried out from the sum of expression contained in these sections. Therefore the results show total PVA, PVN and SON expression. ‘The Mouse Brain in Stereotaxic Coordinates’ (Paxinos, 1996) was used as a guide to confirm the identity of the structure being examined. Due to the thickness of the sections, the stained nuclei appeared in different focal planes. To solve this problem the counting was carried out twice for the same location using a different focus. When counting, the nuclei were marked with a cross using the computer program tool, therefore avoiding double-counting the neurones when changing the focus.

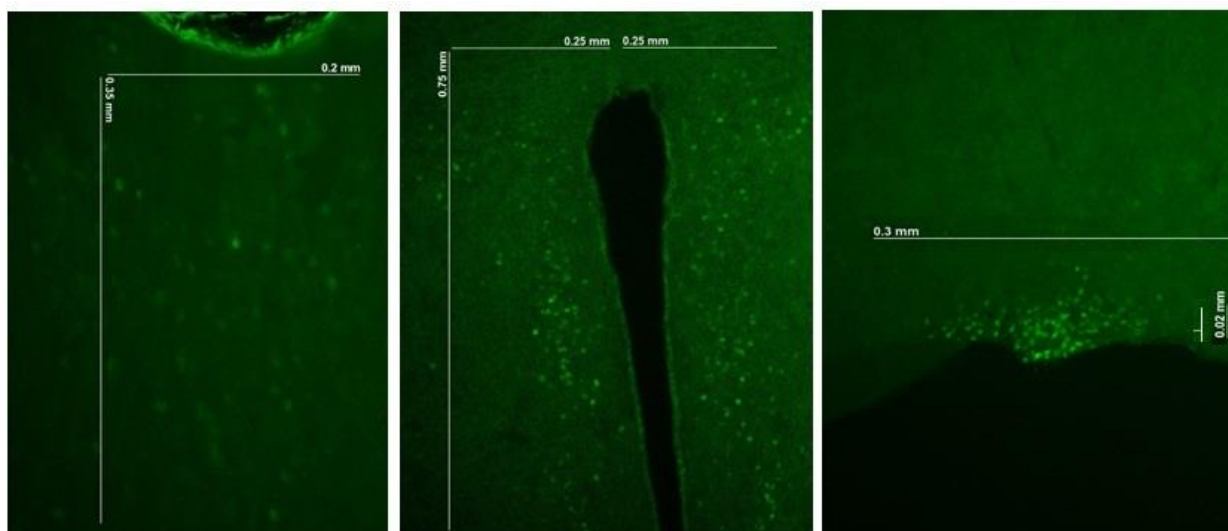


Figure 29 - Surface area counted for c-fos expression. Paraventricular thalamic nucleus, anterior PVA: 0.35 mm x 0.2 mm (A), paraventricular nucleus of hypothalamus PVN: 0.75 mm x 0.25 mm for each side (B) and supraoptic nucleus SON: length of area 0.3 mm and 0.02 width.

5.4.6 Statistical analyses

The statistical analyses was carried out for the forebrain areas, comparing the total number of Fos-positive nuclei with one way ANOVA for PVA, PVN, SON compared to vehicle (control). Multiple Comparisons versus Control Group (Holm-Sidak method): Overall significance level = 0.05. SigmaPlot 11.

5.5 Results

The immunohistochemistry analyses of the forebrain and midbrain showed the presence of Fos-positive neurones. However, in the midbrain Fos expression was not consistent and the signal was very small, which would make the counting potentially inaccurate. For this reason it was decided only to present qualitative analyses indicating the parts of the midbrain where the Fos protein was expressed. In the midbrain sections the Fos expression was observed for all treatments, but not for all individuals in the group (Table 38), and expression was located in the midbrain areas of dorsomedial periaqueductal grey (DMPAG), dorsal lateral periaqueductal grey (DLPAG) and ventrolateral periaqueductal grey (VLPAG) (Figure 30).

The forebrain sections showed more consistent expression of Fos-positive neurones and for this reason it was possible to carry out quantitative analyses. Figure 31, Figure 32 and Figure 33 show the regions analysed for the forebrain, which are the ventral regions of the paraventricular thalamic nucleus, anterior (PVA) (Figure 31), paraventricular nucleus of hypothalamus (PVN) (Figure 32) and the supraoptic nucleus (SON) (Figure 33). In each case expression was quantified for both sides of the brain. Analyses of Fos-positive neurones in these parts showed a significant decrease of the expression of cells in the paraventricular nucleus of hypothalamus (PVN) for the groups treated with *H. crenata* 150 mg/kg (p.o.) after being through the acetic acid test (Figure 35, Table 39 and Table 40). This reduction of Fos protein in the PVN is illustrated in Figure 37 where it shows the difference in Fos expression when comparing mice treated with vehicle, HC 15 mg/kg (p.o.) and HC 150 mg/kg (p.o.). However, for the other brain parts, PVA and SON, there was no significant difference in the level of expression when compared with the vehicle (Table 41 and Table 42).

Treatments	N	PAG area (number of mice with detectable Fos expression)			
		DMPAG	DLPAG	LPAG	VLPAG
Vehicle	8	3	5	5	6
HC 15 mg/kg	8	1	5	4	4
HC 150 mg/kg	8	1	6	5	6

Table 38 - Qualitative analysis of Fos expression observed in the mouse midbrain.

Analysis in areas of dorsomedial periaqueductal grey (DMPAG), dorsal lateral periaqueductal grey (DLPAG) and ventrolateral periaqueductal grey (VLPAG), indicating number of mice with detectable Fos expression.

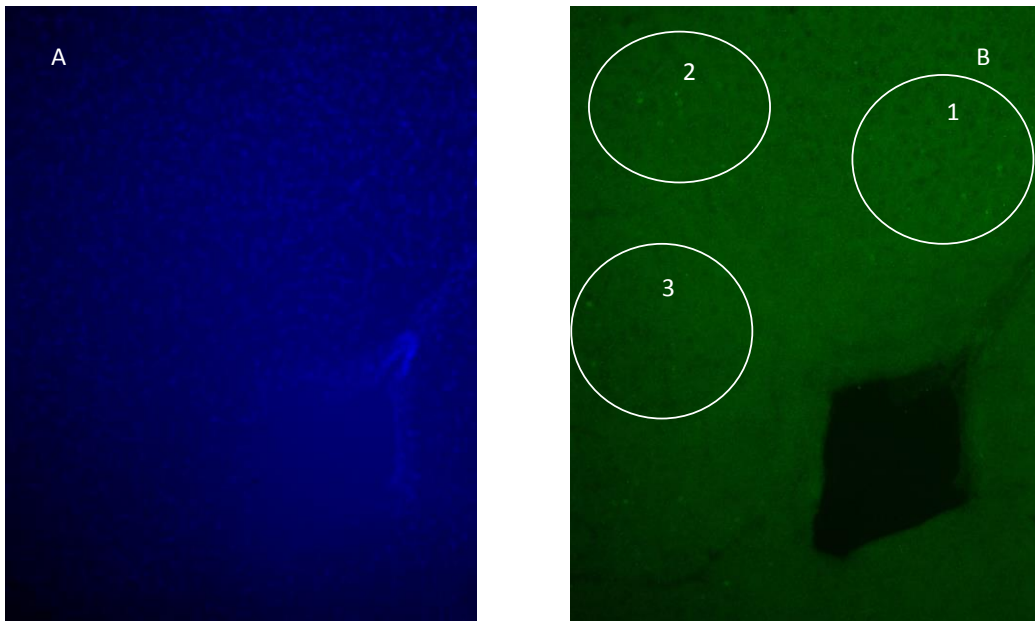
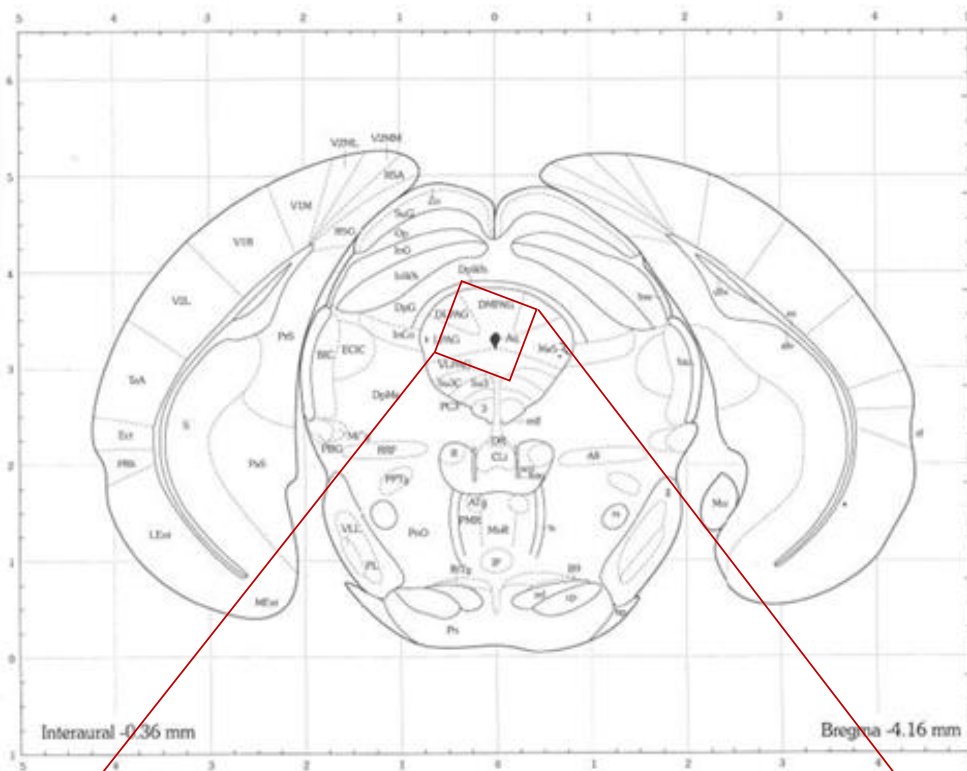


Figure 30 – Photomicrograph of midbrain areas. Photomicrograph of DAPI nuclear staining (A) and c-fos protein expression (B) observed in the DMPAG (1), DLPAG (2), LPAG (3) and VLPAG (4) FITC labelled (10 x magnification)

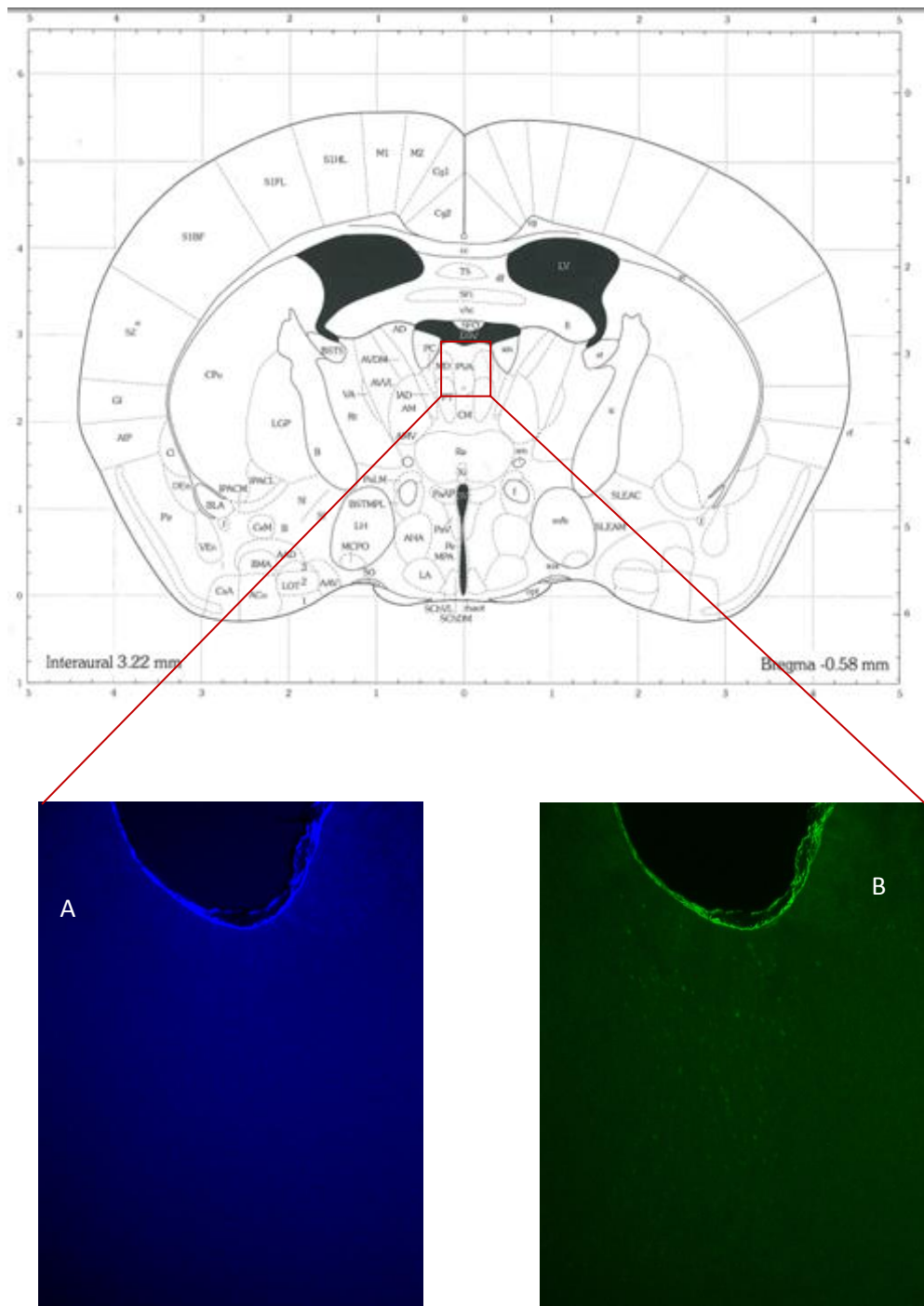


Figure 31 - Photomicrograph of paraventricular thalamic nucleus, anterior (PVA). DAPI nuclear staining (A) (10x magnification) and c-fos protein expression FITC labelled (B) (10x magnification) from the paraventricular thalamic nucleus, anterior (PVA)

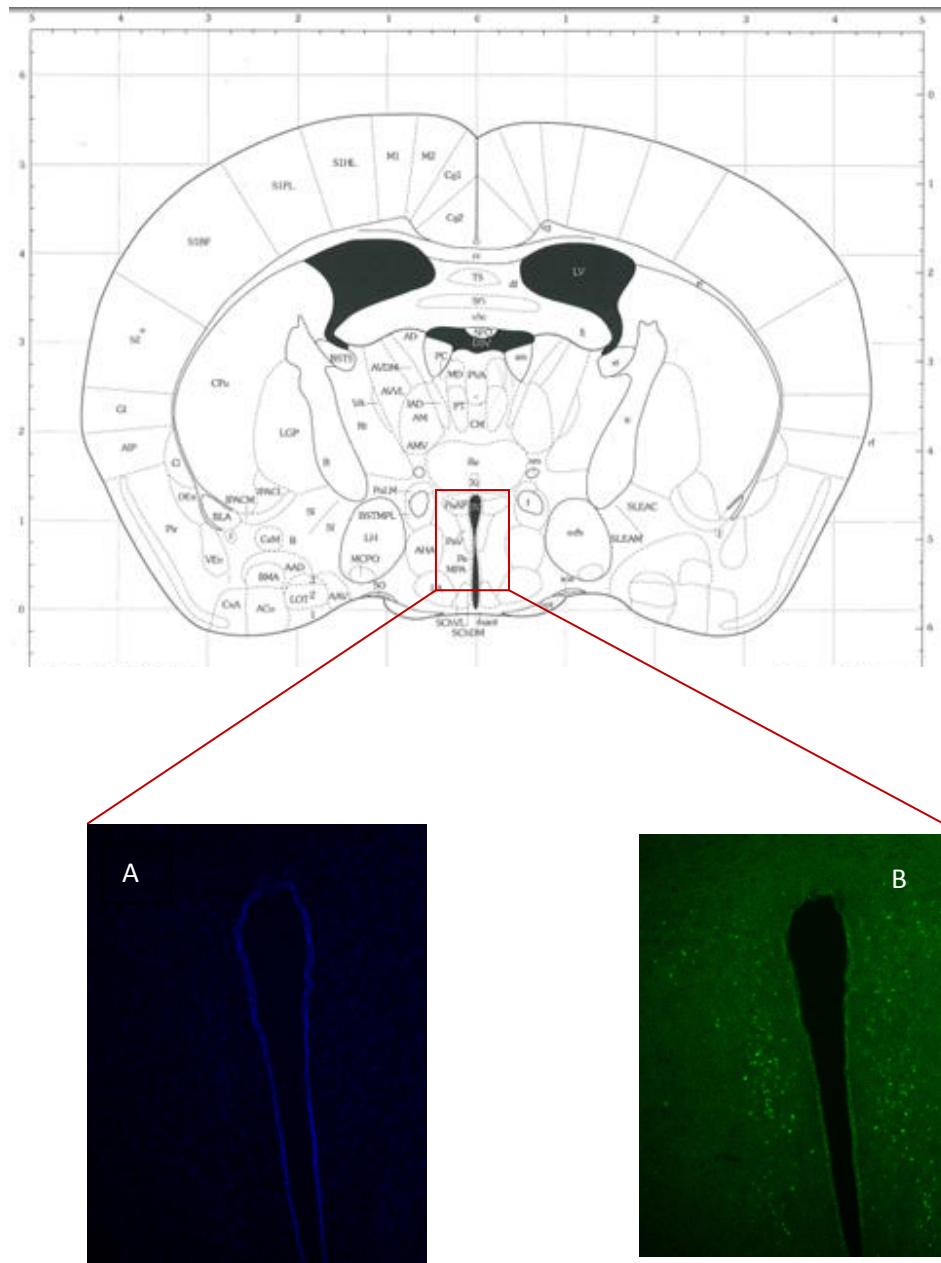


Figure 32 - Photomicrograph of paraventricular nucleus of the hypothalamus (PVN). DAPI nuclear staining (A) (10x magnification) and c-fos protein expression FITC labelled (B) (10x magnification) from the Paraventricular nucleus of the hypothalamus (PVN)

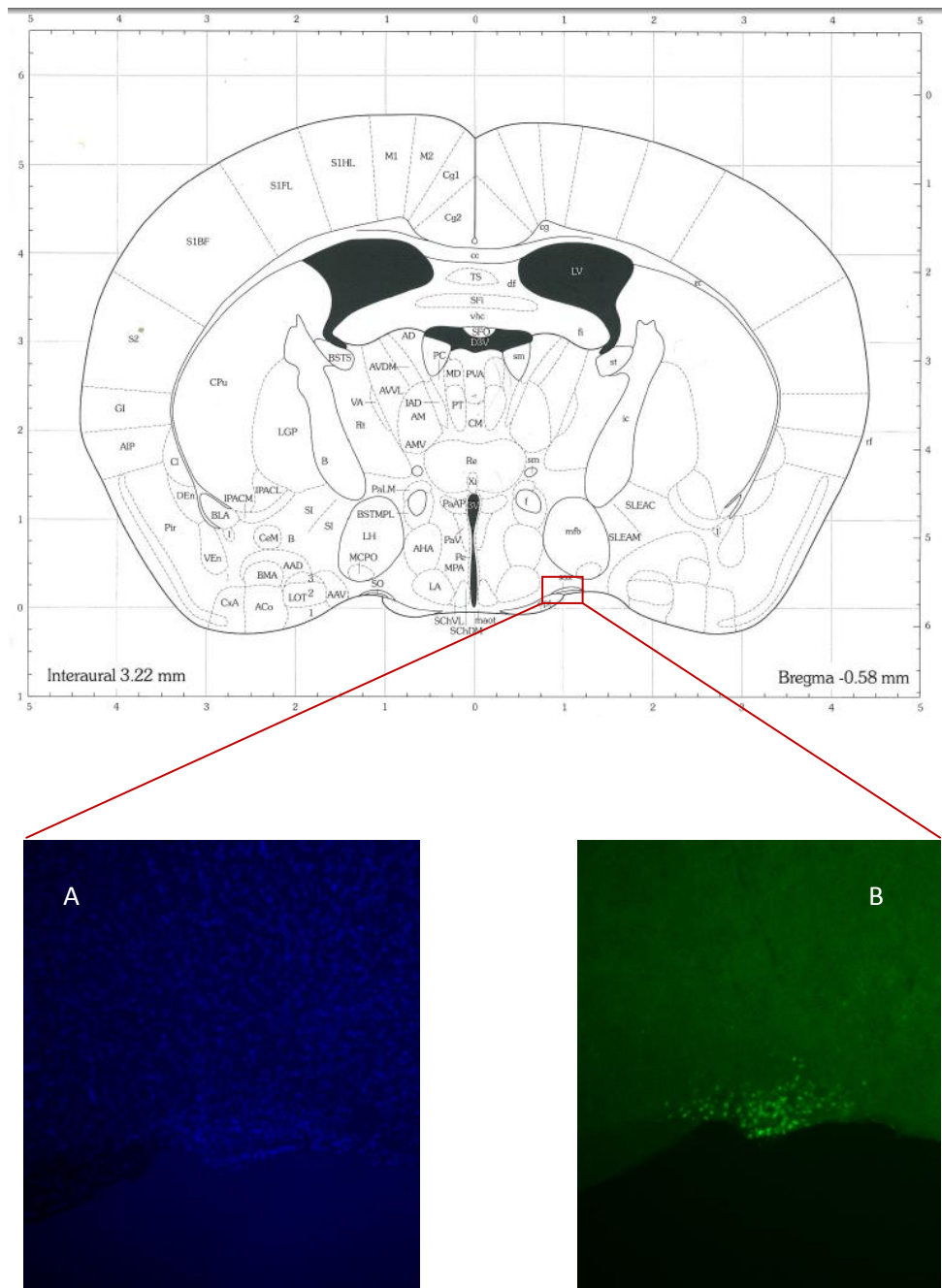


Figure 33 - Photomicrograph of supraoptic nucleus (SON). DAPI nuclear staining (A) (10x magnification) and c-fos protein expression FITC labelled (B) (10x magnification) from the supraoptic nucleus (SON)

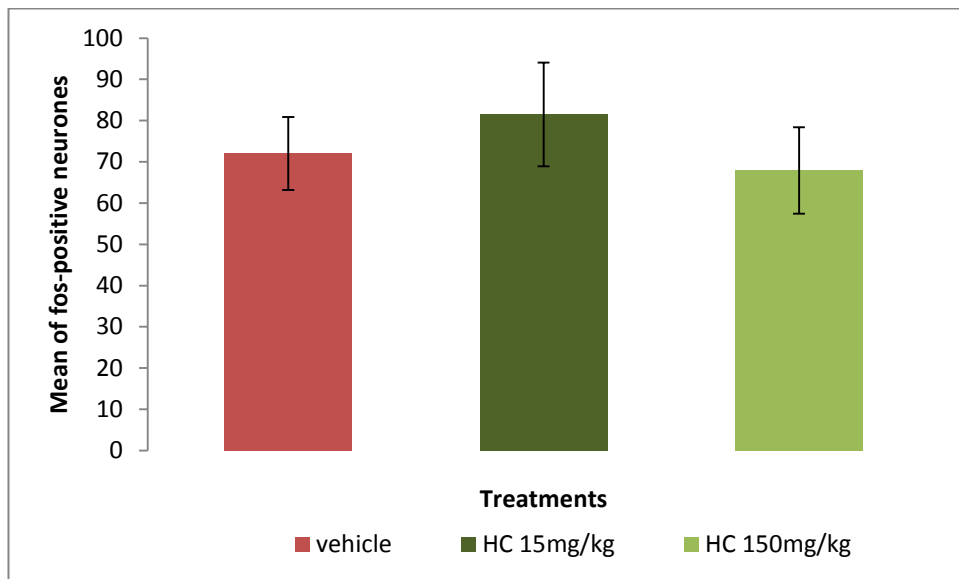


Figure 34 – Number of c-fos positive neurons in the forebrain paraventricular thalamic nucleus, anterior (PVA). Mean \pm S.E.M. of c-fos positive neurons in the forebrain paraventricular thalamic nucleus, anterior (PVA), of mice treated with vehicle (p.o.), *H. crenata* decoction extract dose 15 mg/kg (p.o.) and *H. crenata* decoction extract dose 150 mg/kg (p.o.).

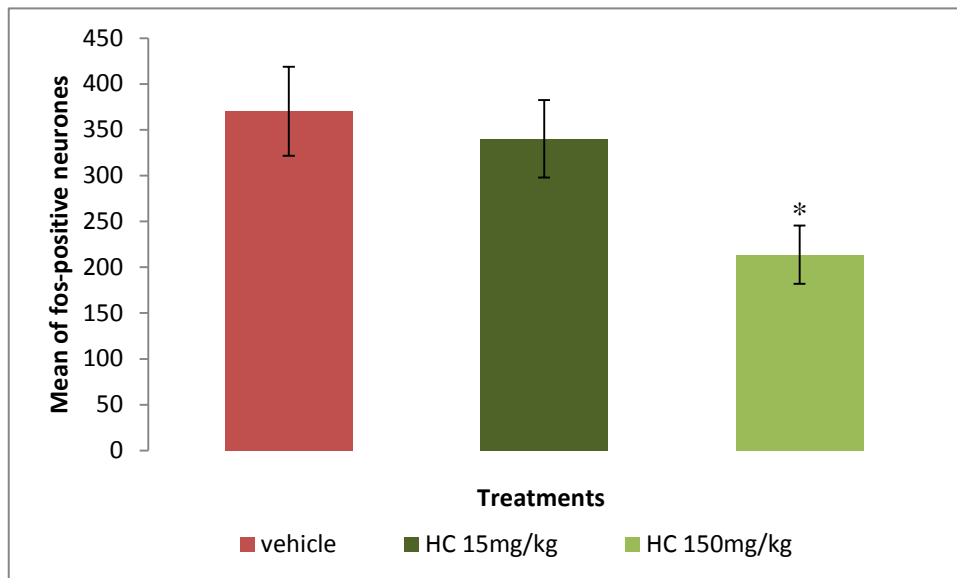


Figure 35 – Number of c-fos positive neurons in the forebrain paraventricular nucleus of the hypothalamus (PVN). Mean \pm S.E.M. of c-fos positive neurons in the forebrain Paraventricular nucleus of the hypothalamus (PVN), of mice treated with vehicle (p.o.), *H. crenata* decoction extract dose 15 mg/kg (p.o.) and *H. crenata* decoction extract dose 150 mg/kg (p.o.). * Analysis of Variance (ANOVA), multiple comparisons versus vehicle (Holm-Sidak method): Overall significance level = 0.05. SigmaPlot 11.

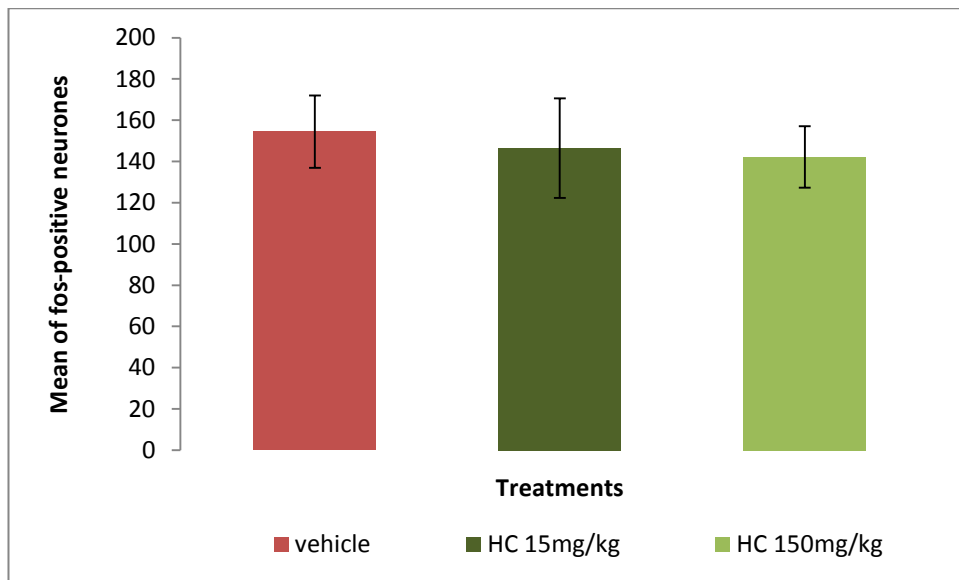


Figure 36 – Number of c-fos positive neurons in the forebrain supraoptic nucleus (SON). Mean \pm S.E.M. of c-fos positive neurons in the forebrain supraoptic nucleus (SON), of mice treated with vehicle (p.o.), *H. crenata* decoction extract dose 15 mg/kg (p.o.) and *H. crenata* decoction extract dose 150 mg/kg (p.o.).

Treatment comparison	Diff of Means	t	Unadjusted P	Critical Level
Vehicle vs. HC 150 mg/kg	156.5	2.668	0.014	0.025
Vehicle vs. HC 15 mg/kg	29.875	0.509	0.616	0.05

Table 39 - Multiple comparisons statistic of one way ANOVA for analyses of Fos expression in the PVN of mice after acetic acid injection. Animals treated with *H. crenata* (HC) decoction extract 15 and 150 mg/kg (p.o.) compared with vehicle (p.o.).

Source of Variation	DF	SS	MS	F	P	Power
Between Groups	2	110449.8	55224.875	4.014	0.033	0.515
Residual	21	288932.3	13758.679			
Total	23	399382				

Table 40 - Output of one way ANOVA for analyses of c-fos expression in the PVN of mice after acetic acid injection. Animal groups treated with *H. crenata* (HC) decoction extract 15 mg/kg (p.o.) and HC 150 mg/kg (p.o.) compared with vehicle (p.o.).

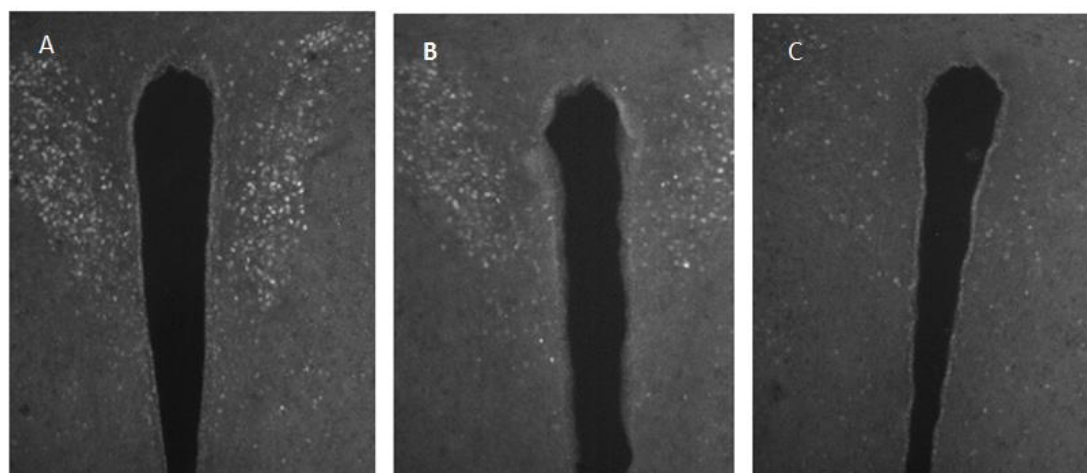


Figure 37 - Effect of *H. crenata* decoction extract on Fos protein expression in the paraventricular nucleus of the hypothalamus (PVN) after noxious stimuli of acetic acid. Groups treated with vehicle (A), HC 15 mg/kg (B) and HC 150 mg/kg (C).

Source of Variation	DF	SS	MS	F	P	Power
Between Groups	2	0.0248	0.0124	0.422	0.661	0.049
Residual	21	0.617	0.0294			
Total	23	0.642				

Table 41 - Output of one way ANOVA for analyses of c-fos expression in the paraventricular thalamic nucleus, anterior (PVA) area of mice after acetic acid injection. Animal groups treated with *H. crenata* (HC) decoction extract 15 mg/kg (p.o.) and HC 150 mg/kg (p.o.) compared with vehicle (p.o.). All data points are n=8.

Source of Variation	DF	SS	MS	F	P	Power
Between Groups	2	0.0129	0.00644	0.186	0.832	0.049
Residual	21	0.729	0.0347			
Total	23	0.741				

Table 42 - Output of one way ANOVA for analyses of c-fos expression in the SON area of mice after acetic acid injection. Animal groups treated with *H. crenata* (HC) decoction extract 15 mg/kg (p.o.) and HC 150 mg/kg (p.o.) compared with vehicle (p.o.). All data points are n=8.

5.6 Discussion

The aim of this chapter was to investigate if the *H. crenata* decoction extract that previously showed significant effects in reducing writhing in the acetic acid test could also reduce the expression of Fos in specific areas of the midbrain and forebrain.

In the midbrain, Fos expression was observed for all treatments, in a small number of neurones located in the dorsal lateral periaqueductal grey (DLPAG), dorsomedial periaqueductal grey (DMPAG) and ventrolateral periaqueductal grey (VLPAG). Observation of Fos in the periaqueductal grey was also reported by a number of groups (Snowball *et al.*, 2000; Bianchi *et al.*, 2003; Mitsui *et al.*, 2003) using acetic acid to induce pain. In their study of Fos expression in the periaqueductal grey (PAG) of rats, Keay and Bandler (1993) described that deep noxious stimulation can lead to fos-positive cells in the VLPAG while superficial noxious stimulation can lead to fos-

positive cells predominantly in the LPAG. Therefore we could suggest that, based on this information, the c-fos neurons present in the midbrain PAG ventrolateral areas of the mice in the present study indicated that they suffered deep noxious stimulation.

In the forebrain, Fos expression was observed in the paraventricular thalamic nucleus, anterior part (PVA), paraventricular nucleus of hypothalamus (PVN) and supraoptic nucleus (SON). According to Ab Aziz and Ahmad (2006) the thalamus has a specialised structure that connects with multiple ascending pain pathways. It is involved in processing of nociceptive information before transmitting the information to various parts of the cortex. The hypothalamus is also involved in the sensory discrimination and motivational components of pain, and has substantial importance in modulation of stress activity (Fontes *et al.*, 2011; Peter, 2011; Ueta *et al.*, 2011). This study showed that the level of Fos expression in the PVA and SON was not affected by *H. crenata* extract. However, the higher dose of *H. crenata* extract significantly reduced Fos expression in the PVN (43% reduction). Thus, it is possible that *H. crenata* compounds could be decreasing the visceral pain that is transmitted to the PVN. However, the decrease of Fos in the PVN area was not significant for the lower dose of HC (15 mg/kg) that was effective in reducing writhing during the acetic acid test. The fact that PVN is also involved in stress activity could suggest that this c-fos decrease could be due to a potential anxiolytic effect induced by a particular compound that requires a higher dose in order to be active (Gao *et al.*, 2010).

Regarding conventional anaesthetic or other antinociceptive drugs acting to reduce c-fos in the PVN, there are several studies reporting such effects, including the results reported by Peng *et al.* (2009) described in the introduction of this chapter. The Peng group observed that *Ligustici chuanxiong* Hort essential oil was effective in reducing Fos expression in the brain stem and hypothalamus of mice that demonstrated an antinociceptive effect in the acetic acid inducing writhing test. However examination of the photomicrographs of Fos-immunoreactivity suggests that their data may have been compromised by an artefact of staining of red blood cells which contain a high level of endogenous peroxidase activity and can appear as a series of dark “immunoreactive” elements closely lined up. Therefore, the present study is the first to accurately measure the effect of a phytochemical on nociceptive responses using Fos expression. The study by Peng *et al.* (2009) also reported the effect of the treatment on prolonging the sleeping time induced by sodium pentobarbital, therefore suggesting that the plant compounds

also have sedative effect. The substances opiates, ethanol, cannabinoids, nicotine, cocaine and amphetamines have been described for inducing activation of the hypothalamic-pituitary-adrenal axis, with the consequent release of adrenocorticotrophic hormone and glucocorticoids (Armario, 2010). Regarding reports about Fos expression in hypothalamic regions by cocaine and ethanol, contrary to *H. crenata* 150 mg/kg (p.o.), cocaine and ethanol increases c-fos expression in this forebrain area (Torres and Rivier, 1992; Westerman *et al.*, 2010). Local anaesthetics, like lidocaine and bupivacaine, were reported for their inability to reduce c-fos expressed in the PVN after surgery, despite being able to reduce c-fos expression in the spinal cord (Stenberg *et al.*, 2005). This could indicate that although the *H. crenata* extract lower dose (HC 15 mg/kg) did not reduce c-fos in the PVN area it could be reducing c-fos in the spinal cord area. However, because the spinal cord was not analysed in this study, this hypothesis could not be examined.

Fos expression in the PVA and SON were unaffected by treatment with HC extract. Regarding the function described for these structures, there is mention that the PVA is involved in central sleep and circadian regulation (Salazar-Juarez *et al.*, 2002) and its activation by acetic acid may relate to an arousal effect of this nociceptive agent. The SON is largely known for its role in water balance, through the release of vasopressin, and the control of sodium excretion (natriuresis) through release of oxytocin. It is likely that acetic acid represents an osmotic stimulus as well as a stress and pain stimulus that may underlie its activation. In relation to the SON, studies in rats described its functional connections with the stomach. In relation to antinociception it was reported that SON plays an important role in pain modulation and the hormone oxytocin secreted is involved in this effect (Lu *et al.*, 2011; Yang *et al.*, 2011). However it seems that the antinociceptive effects observed for *H. crenata* are not in connection with the pathway that would affect c-fos expression in these structures.

The fact that these areas analysed, especially the PVN, are involved in multifunction activities raises doubt as to whether the c-fos expression observed was due to the pain induced by the acetic acid. This expression could be related to several activities, mainly because c-fos is not uniquely expressed in the pain condition; therefore this experiment would have more robust results if the experiment design had a group that did not receive the noxious stimulus by acetic acid.

5.7 Conclusions

In conclusion, the present study demonstrated that, compared to the vehicle group, mice treated with *H. crenata* decoction extract at 150 mg/kg (p.o.) showed decreased c-fos protein expression in the paraventricular nucleus of the hypothalamus (PVN) following injection of acetic acid, a model of visceral nociception. This significant reduction of c-fos expression was not observed for the lower dose 15 mg/kg (p.o.).

So far, there is no conclusive study of a conventional antinociceptive drug that is effective in reducing c-fos expression in the PVN area. Therefore, the fact that there was a trend for dose dependence for c-fos reduction from *H. crenata* doses, but not a significant effect of c-fos reduction from 15 mg/kg that was effective in writhing reduction, could suggest that the c-fos reduction observed may not be due to antinociception but to another activity, probably anxiolytic or sedative.

**Chapter 6. Analysis of COX Inhibition Activities from *Hyptis crenata*
Decoction Extract**

6.1 Introduction

Some analgesic drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) have their effect through inhibition of the fatty acid cyclo-oxygenase enzyme (COX). The different variety of COX enzyme and its mechanisms on pain relief are described in Chapter 1.

In order to investigate if the *H. crenata* antinociceptive effect is also related to COX inhibition, a COX inhibitor assay was performed. In this assay inhibition of COX-1 and 2 was analysed.

6.2 Material and methods:

6.2.1 COX Inhibitor Screening Assay

The samples were evaluated with a commercial kit, the Cayman chemical COX Inhibitor screening assay kit, item n° 560131.

According to the Cayman description about this assay, the COX (ovine) inhibitor screening assay directly measures $\text{PGF}_{2\alpha}$ (Prostaglandin $\text{F}_{2\alpha}$) by SnCl_2 (Stannous Chloride) reduction of COX-derived PGH_2 produced in the COX reaction. The prostanoid product is quantified via enzyme immunoassay (EIA) using a broadly specific antiserum that binds to all the major PG compounds. The assay includes both ovine COX-1 and human recombinant COX-2 enzymes.

6.2.1.1 COX reaction procedure

The first step in this assay was to aliquot the background samples in the test tubes (inactivate COX-1 and COX-2) and mix with heme. Next, heme was also added in the 100% COX-1 and COX-2 tubes and the same procedure was carried out for the COX-1 and COX-2 tubes with *H. crenata* extraction. The tubes were incubated for ten minutes at 37°C , the reaction was initiated by adding arachidonic acid to the tubes, and it was vortex and incubated for two min. The next procedure was to add HCl (1M) to the tubes in order to stop the enzyme catalysis, the tubes were removed from the bath and stannous chloride was added to set-up the reaction product and reduce it in a form to be analysed. After, the tubes were incubated for five minutes at room temperature.

6.2.1.2 Enzyme immunoassay procedure (EIA)

Firstly, the EIA buffer, wash buffer and standards were prepared. Then, the background, COX 100% initial activity and HC extract previously prepared in the COX reaction procedure were diluted. The background samples were diluted by adding 10 µl of the previously prepared background samples (Background COX-1 or COX-2) to a tube containing 990 µl of EIA buffer (BC1 or BC2). The BC1 or BC2 tube contained a 1:100 dilution of the original sample.

The COX 100% Initial Activity samples were diluted by adding 10 µl of the previously prepared COX 1 or COX 2 100% Initial Activity sample to 990µl of EIA buffer (IA1). Next 50 µl of IA1 dilution was added to another tube containing 950µl of EIA buffer (IA2). Then, 500 µl of tube IA2 was added to another tube containing 500 µl. Therefore, tube IA2 contained 1:2000 dilution of the original sample and tube IA3 contained 1:4000 dilution of the original sample (IA1).

The COX inhibitor samples (*H. crenata* samples), previously prepared were diluted following the same procedure for COX 100% Initial Activity, however its dilutions were called C1, C2 and C3. The C2 and C3 tubes contained 1:2000 and 1:4000 dilution respectively of the original sample (C1).

The next procedure was to add the samples to the plate well coated with mouse anti-rabbit IgG. The plate wells were composed of blank, non-specific binding, maximum binding, PG standards, background COX-1 and COX-2, 100% initial activity samples and the HC extract dilutions (88 wells, Figure 38). Finally, the AChE tracer and antiserum were added to the specific wells. The Plate was incubated at room temperature for eighteen hours on an orbital shaker, rinsed five times with wash buffer, developed with Ellman's Reagent and read on a microplate reader (Thermo Lab Systems, Multiscan Asent) at 410 nm.

The percent inhibition was calculated using the Cayman analysis tool spreadsheet, the calculation was also checked by Brooke Kilyanek, M.S. Cayman technical support representative.

There were some samples that were not calculated due to high % B/B₀ (%Bound/Maximum Bound). For this reason the number of samples per group is different.

	1	2	3	4	5	6	7	8	9	10	11
A	Blank	Std0 1	Std0 1	Background COX1	Background COX2	COX1 (C2) Hexane fraction	COX1 (C2) DCM fraction	COX1 (C3) Hexane fraction	COX1 (C3) Water fraction	COX2 (C2) Hexane fraction	COX2 (C2) Water fraction
B	Blank	Std0 2	Std0 2	Background COX1	Background COX2	COX1 (C2) Hexane fraction	COX1 (C2) DCM fraction	COX1 (C3) Hexane fraction	COX1 (C3) Water fraction	COX2 (C2) Hexane fraction	COX2 (C2) Water fraction
C	NSB	Std0 3	Std0 3	COX1 (IA2 100% activity)	COX1 (IA3 100% activity)	COX1 (C2) Methanol fraction	COX1 (C2) HC 15mg/kg	COX1 (C3) DCM fraction	COX1 (C3) HC 15mg/kg	COX2 (C2) DCM fraction	COX2 (C2) HC 15mg/kg
D	NSB	Std0 4	Std0 4	COX1 (IA2 100% activity)	COX1 (IA3 100% activity)	COX1 (C2) Methanol fraction	COX1 (C2) HC 15mg/kg	COX1 (C3) DCM fraction	COX1 (C3) HC 15mg/kg	COX2 (C2) DCM fraction	COX2 (C2) HC 15mg/kg
E	Bo	Std0 5	Std0 5	COX2 (IA2 100% activity)	COX2 (IA3 100% activity)	COX1 (C2) Water fraction	COX1 (C2) HC 150mg/kg	COX1 (C3) Methanol fraction	COX1 (C3) HC 150mg/kg	COX2 (C2) Methanol fraction	COX2 (C2) HC 150mg/kg
F	Bo	Std0 6	Std0 6	COX2 (IA2 100% activity))	COX2 (IA3 100% activity)	COX1 (C2) Water fraction	COX1 (C2) HC 150mg/kg	COX1 (C3) Methanol fraction	COX1 (C3) HC 150mg/kg	COX2 (C2) Methanol fraction	COX2 (C2) HC 150mg/kg
G	Bo	Std0 7	Std0 7	COX2 (C3) Hexane fraction	COX2 (C3) DCM fraction	COX2 (C3) Methanol fraction	COX2 (C3) Water fraction	COX2 (C3) HC 15mg/kg	COX2 (C3) HC 150mg/kg	COX2 (C3) Hexane fraction	COX2 (C3) DCM fraction
H	TA	Std0 8	Std0 8	COX2 (C3) Hexane fraction	COX2 (C3) DCM fraction	COX2 (C3) Methanol fraction	COX2 (C3) Water fraction	COX2 (C3) HC 15mg/kg	COX2 (C3) HC 150mg/kg	COX2 (C3) Hexane fraction	COX2 (C3) DCM fraction

Figure 38 - Layout of the samples set on the plate for which the absorbance was measured for the COX inhibition assay. COX 1 and COX 2 inhibitors evaluated (*Hyptis crenata* decoction (HC) samples and HC fractions containing dilution of C2=1:2000 and C3=1:4000), COX1 and COX 2 background (dilution of 1:100), COX 1 and COX 2 100% activity (dilution of 1:2000 and 1:4000), Blank, NSB=non-specific binding, BO=maximum binding, TA=total activity and Std=standards.

6.2.2 *H. crenata* samples concentration

The same decoction extract and fractions of decoction extract that were used in the antinociceptive test carried out on the mice were also used in this assay. The procedures for the *H. crenata* decoction extraction and fractions are described in the methodology section of Chapter 3.

6.2.3 *H. crenata* decoction extract

The concentration used to start the assay and the final concentration applied in the well, presented in Table 43, were based on the same concentration given to the mice in the antinociceptive tests (HC 1500 µg /10 ml and HC 15000 µg /10 ml). However instead of using water as a solvent, the reaction buffer provided in the COX inhibition kit was used.

<i>H. crenata</i> treatments	Start concentration HC µg/10 ml reaction buffer	Final concentration of HC µg per 50 µl applied in the well
Decoction extract lower dose (HCl)	1500 µg	0.00097µg
Decoction extract higher dose (HCh)	15000 µg	0.00978 µg

Table 43 - Start concentration for performing COX reactions and final concentration of *H. crenata* decoction applied in the plate wells in the COX inhibition screening assay

In addition, HC fractions were evaluated, the concentration used to start the assay and the final concentration applied in the well, presented in Table 44, were based on the same concentration given to the mice in the antinociceptive tests (HC Hexane fraction 210 µg/10 ml, HC DCM fraction 400 µg/10 ml, HC Methanol 1480 µg/10 ml and HC Water fraction 2300 µg/10 ml). The water and Tween 80 used as a solvent in the animal experiment were substituted by the reaction buffer provided in the COX inhibition kit.

H. crenata treatments, Fractions/Solvent	Start concentration HC fractions µg/10 ml reaction buffer	Final concentration of HC µg per 50 µl applied in the well
Hexane fraction	210 µg	0.00013 µg
DCM fraction	400 µg	0.00026 µg
Methanol fraction	1480 µg	0.00096 µg
Water fraction	2300 µg	0.0015 µg

Table 44 - Start concentration for performing COX reactions and final concentration of *H. crenata* decoction fractions applied in the plate wells in the COX inhibition screening assay

The data were analysed using one-way ANOVA and when the overall P-value was <0.05 Tukey's multiple pairwise comparison test was used to test if any of the results for any extracts or fractions were different from others.

6.3 Results

In general the results of this assay showed imprecise data due to problems with the sensitivity of the test that was caused by the samples' dilution (too diluted) and low inhibition by the positive control 100% initial activity. The problem was mainly observed for the evaluation of COX-1 inhibition where the ANOVA was non-significant (Table 45) and none of the samples presented any difference from the control value (see Figure 39). If anything the lower HC concentration, hexane, DCM and methanol fractions seemed to be activating COX-1 instead of inhibiting it.

Source of Variation	DF	SS	MS	F	P	Power
Between Groups	6	33665.91	5610.985	1.141	0.381	0.075
Residual	17	83602.31	4917.783			
Total	23	117268.2				

Table 45 - Multiple comparisons statistic of one way ANOVA for COX-1 inhibition.

Control group (100% initial activity) n=4 versus *H. crenata* decoction 1500 µg/ml (HCl) N=3, *H. crenata* decoction 15000 µg/ml (HCh) N=3 and *H. crenata* decoction fractions hexane 210 µg/ml (HexFrac) N=4, DCM fraction 400 µg/ml (DCMFrac) N=3, methanol fraction 1480 µg/ml (MetFrac) N= 4 and water fraction 2300 µg/ml (WatFrac) N=4. One way ANOVA (Holm-Sidak method), overall significance level = 0.05.

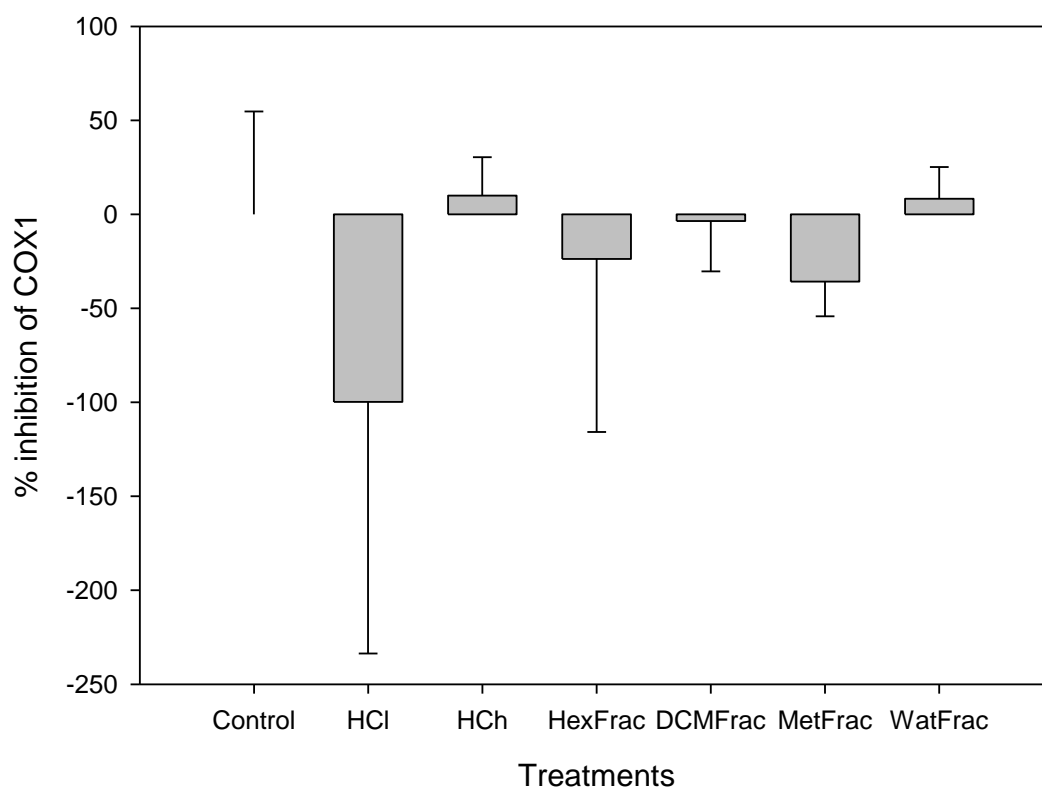


Figure 39 – Effect of HC decoction fractions on COX-1 inhibition. Mean+ standard deviation of COX-1 inhibition for the control (100% initial activity) n=4, *H. crenata* decoction 1500 µg/ml (HCl) n=3, *H. crenata* decoction 15000 µg/ml (HCh) n=3 and *H. crenata* decoction fractions hexane 210 µg/ml (HexFrac) n=4, DCM fraction 400 µg/ml (DCMFrac) n=3, methanol fraction 1480 µg/ml (MetFrac) n= 4 and water fraction 2300 µg/ml (WatFrac) n=4.

Regarding COX-2 the ANOVA did show significance (Table 46), so even though none of the samples showed difference from the control, all *H. crenata* treatments seemed to inhibit COX-2 but it is not possible to suggest which of the samples is more effective or if there is a dose dependence effect from the higher and lower dose of the decoction (Figure 40). For example, while the *H. crenata* decoction extracts in a start concentration of 1500 µg inhibited 41% of COX-2 and the higher concentration 15000 µg inhibited 83%, as shown in Figure 40, the standard deviations were too high to distinguish between the two doses. The corresponding inhibition values for the fractions were: hexane (61%), DCM (56%), methanol (72%) and water (63%).

Source of Variation	DF	SS	MS	F	P	Power
Between Groups	6	13731.2	2288.533	3.31	0.046	0.525
Residual	10	6914.477	691.448			
Total	16	20645.68				

Table 46 - Multiple comparisons statistic of one way ANOVA for COX-2 inhibition.

Control group (100% initial activity) N =4 versus *H. crenata* decoction 1500 µg/ml (HCl) N=3, *H. crenata* decoction 15000 µg/ml (HCh) N=3 and *H. crenata* decoction fractions hexane 210 µg/ml (HexFrac) N=4, DCM fraction 400 µg/ml (DCMFrac) N=5, methanol fraction 1480 µg/ml (MetFrac) N = 3 and water fraction 2300 µg/ml (WatFrac) N=2. One way ANOVA (Holm-Sidak method), overall significance level = 0.05.

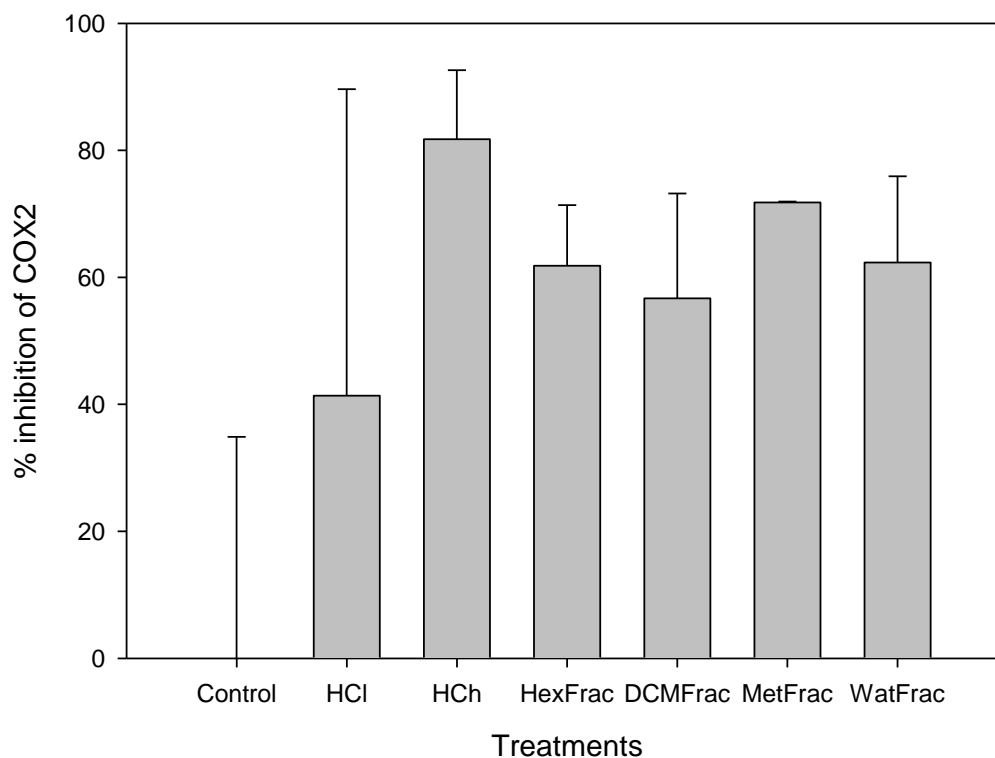


Figure 40 - Effect of HC decoction fractions on COX-2 inhibition. Mean+ standard deviation of COX-2 inhibition for the control (100% initial activity) N=4, *H. crenata* decoction 1500 µg/ml (HCl) N=3, *H. crenata* decoction 15000 µg/ml (HCh) N=3 and *H. crenata* decoction fractions hexane 210 µg/ml (HexFrac) N=4, DCM fraction 400 µg/ml (DCMFrac) N=5, methanol fraction 1480 µg/ml (MetFrac) = 3 and water fraction 2300 µg/ml (WatFrac) N=2.

6.4 Discussion

The aim of this experiment was to investigate whether *H. crenata* compounds that showed to have demonstrated antinociceptive properties would affect prostaglandin levels in a COX inhibitor screening assay. Despite the problems with the sensitivity of this assay, the results indicate that *H. crenata* treatments may be effective to inhibit COX-2, while the results for COX-1 showed that none of the fractions or extracts had any inhibitory effect, although for most of them some of the samples showed negative inhibition. According to an explanation from a Cayman technician, the negative inhibition values may mean that the *H. crenata* compound is actually activating COX-1.

However, it may also mean that there was something in the *H. crenata* decoction sample that interfered with the assay. There are no reports in the literature about *Hyptis* increasing or decreasing COX-1 activity.

In contrast, the inhibition of COX-2 seems to be more plausible, and there are reports about *Hyptis* compounds inhibiting COX-2 activity (Kuhnt *et al.*, 1995b; Raymundo *et al.*, 2011). However, all *H. crenata* fractions induced this effect at a similar level, in contrast to the antinociceptive results observed in the animal test which shows that hexane fraction was the most active fraction and DMC fraction, which obtained COX inhibition value of 56% in this assay, was inactive in the antinociceptive tests. There are two possible explanations for this result; firstly this assay may not have been sensitive enough to detect the difference between the fractions. It is also possible that even if compounds with COX-2 inhibition activity are present in the extracts and fractions, the antinociceptive properties observed for *H. crenata* in the in vivo experiment may not be due to COX-2 inhibition, or at least this may not be the main mechanism responsible for the effect, for example if COX-2 inhibitors have low oral bioavailability or are degraded during digestion.

When considering the possibility of all fractions to inhibit COX-2, it could indicate that there are several *H. crenata* compounds that are able to induce this effect or that the effect is produced by one particular compound that is present in all decoction fractions. The compounds that appear in all decoction fractions and could be involved in the COX - 2 inhibition are: rosmanol isomer n° 53, carnosic acid n° 66, methyl epirosmanol n° 71 and 72 (Chapter 7, Table 48). Besides, some of these compounds, carnosol and carnosic acid, have already been reported for inhibiting COX-2 (Lai *et al.*, 2009; Bauer *et al.*, 2012).

Other compounds that are present in the decoction extract and distributed in its fractions have been reported for inhibiting COX-2, these compounds are vicenin-2 isomer, apigenin isomer, scutellarein *O*-methyl ether isomer, and carnosol isomer (Kim *et al.*, 2004; Lai *et al.*, 2009; Dos Santos *et al.*, 2010; Suou *et al.*, 2011; Bauer *et al.*, 2012; Oh *et al.*, 2012). It is possible that the compound inhibiting COX-2 in these non-antinociceptive active fractions could be compounds that have low bioavailability and absorption in the animal gut, but could be effective when evaluated in the COX inhibition *in vitro* assay (Hu, 2007).

Regarding the COX-1 activation, supposing that it actually occurred, there is a possible explanation for this activity based in the results of the traditional use survey (Chapter 2). The main use of *H. crenata* decoction was for stomach pain, this could indicate that there are compounds in the extract that are increasing COX-1 and enhancing endogenous prostaglandin production, since it has been shown in the literature that it induces gastric protection (Kleine *et al.*, 1993). Therefore, it could contribute to the stomach pain relief, and this could also explain the gastroprotective effects reported for the species *H. spicigera* and *H. mutabilis* (Barbosa and Ramos, 1992; Takayama *et al.*, 2011). In addition, if considering that this COX 1 activation would be occurring, the COX assay result presented in this thesis suggested that the majority of these theoretically gastroprotective compounds, or the most active compounds for this effect, may be in the hexane, DCM and methanol fractions.

Although these results for COX-1 should be interpreted with caution as the findings might not be reliable, the fact that there is an indication that *H. crenata* inhibits COX-2 and not COX-1, could indicate a positive potential for *H. crenata*, since there is a great interest in COX-2 selective compounds for clinical use (Baykal and Gök, 2002). However, it is important to emphasize that overall, the COX assay result presented in this thesis is very preliminary and might not be reliable, therefore it should be repeated.

6.5 Conclusions

The results for this assay suggest that *H. crenata* extract dose at concentration of 1500 µg/ml and 15000 µg/ml and its decoction fractions may be inhibitors of COX-2. In addition, the extract and mainly the water fractions could be increasing COX-1 activities. However, it may also mean that there are compounds in the sample that are interfering with the assay and giving false-positive results, for this reason a repetition of this experiment would be highly recommended to confirm this results.

Chapter 7. Chemical analyses of *Hyptis crenata* decoction and its fractions

7.1 Introduction

As described in Section 1.4.4, the only chemical information reported about *H. crenata* species is related to its volatile compounds. However for the studies reported in this thesis the volatile compounds were not tested since the treatments used were from lyophilised hot water extract and the fractions were also prepared from this lyophilised extract which had lost almost all the volatile compounds present in the plant material. Other reasons for not evaluating the extract with volatile compounds was that the volatiles have been reported to have considerable variation according to the place that *Hyptis* plants grow, as described in the traditional use chapter (Section 2.3.3). In addition it was reported in the traditional use survey that the plant could be collected at any time of the year. Together with information about the common preparation methods (which do not retain the volatiles) this indicates, that the volatile compounds were not crucial for the plant's possible antinociceptive effects. Evaluation of the lyophilised extract and its fraction on two animal models for antinociceptive response demonstrated antinociceptive effects for the decoction extract and the hexane fraction had enhanced bioactivity. In order to find out which chemical compounds were present in the *H. crenata* decoction extract and its fractions, chemical analysis was carried out using liquid chromatography–mass spectrometry (LC-MS).

7.2 Material and methods

7.2.1 LC-MS

The analysis was carried out by Dr Melanie Jayne Howes and her plant chemistry analytical team at Jodrell Laboratory in Kew Gardens, Richmond, Surrey. The equipment used was a High-performance liquid chromatograph machine, thermo Scientific Surveyor and Mass spectrum Pump (HPLC-MS), Thermo Scientific 'LTQ-Orbitrap XL' Linear Ion Trap-Orbitrap, with full scan m/z 250-2000, equipped with an electrospray ionisation unit (positive model). The LC column was a Phenomenex Luna C18, 150 mm x 3 mm, 3 μ m. The column was eluted by using gradient of water : methanol : acetonitrile containing 1% formic acid [400 μ l/min: 90:0:10 (t=0min), 0:90:10 (t=20min), 0:90:10 (t=25min), 90:0:10 (t=27min), 90:0:10 (t=30min)], column temperature 30°C.

For the analyses, *H. crenata* decoction lyophilised extract and its dried fractions (hexane fraction, DCM fraction, methanol fraction and water fraction) were used. Due to the potential for some solvents to affect the LC column and chromatography, dichloromethane and hexane were not used to reconstitute the HC decoction fractions; 100% water was also not used to reconstitute the HC decoction and HC decoction fractions, due to the potential for microbial contamination prior to analysis. Concentrations were based on the amount available and solubility. The preparation details were as shown in Table 47. The injection volume for all samples tested was 5µl.

Extract / fraction	solvent	concentration (mg/ml)
HC decoction extract	50% methanol	10
HC hexane fraction	70% ethanol	4
HC DCM fraction	70% ethanol	9
HC methanol fraction	100% methanol	6
HC water fraction	50% methanol	10

Table 47 - Concentration of HC treatments analysed in the HPLC-MS

The result of the hexane fraction was chosen for comparing with the decoction extract in order to find out which compounds were enriched after the fractionation process. To make this comparison the chromatograms graph (Figure 41 and Figure 42) and the detection table report (Table 48) were used. The reason for selecting the hexane fraction rather than the other fractions is because it was the most active which gave a maximum bioactivity by weight of dry material, as shown in the Hargreaves test and acetic acid test results.

7.2.2 Calculation of compound concentration and enrichment %

The compounds' peaks were measured from chromatograms printed on A3 size paper, the areas under the peaks were calculated considering the shape of each peak as a triangle, therefore the area was calculated as $\frac{1}{2} \times \text{base} \times \text{height}$. When more than one compound was present within a single peak due to them having the same or similar retention times, and they were therefore not distinguishable from each other, the total area of the peak was divided by the number of compounds in order to obtain an estimate. In order to calculate the % of weight, the molecular weights were corrected for cations

(Na⁺, NH₄⁺ or H⁺), and then the proportion of weight was calculated (=compound area/total area of compounds x compound molecular weight). The enrichment of the fraction was calculated as = (amount of extract /amount of fraction) x (concentration of compound in the fraction/concentration of compound in the extract).

7.3 Results

In total 91 compounds, including their isomers, were detected from the HPLC-MS analyses for HC decoction extract and its fractions. In the decoction extract 88 compounds were detected. For the fractions; 16 compounds were detected in the hexane fraction, 31 compounds in the DCM fraction, 53 compounds in the methanol fraction and 25 compounds in the water fraction (Table 48).

As shown in Table 48, the five most prevalent compounds in the decoction were the diterpene trilobiol (n° 64) which accounted for 30.4% of the total mass, the isomers of phenolic terpene methyl epirosmanol (n° 71,72,74,75 and 80; 17,5% of mass), the c-glycosylflavonoid, isoshaftoside (n° 10; 4.5% of mass) and an unidentified diterpenoid (n° 58; 4.4% of mass). In the hexane fraction the major compounds were: the isomers of phenolic compound carnosol/hyptol (n° 76,78 and 84; 34.5% of mass), methyl rosmanol isomers (n° 71 and n° 72; 30.6%), rosmanol and its isomers (n° 52,53,63;15.6% of mass), and isomers of a derivative of hydroxylabdanolidoic acid (n° 83 and 85; 18% of mass). When comparing the HC decoction fraction with the hexane fraction, there are some compounds that were enriched with the hexane fractionation process. The compound phenolic diterpene (n° 84) was the most enriched with this fractionation, since in the decoction results it appears as trace and in the hexane chromatogram it is one of the major compounds. Another compound enriched by this process was hydroxylabdanolidoic acid derivative (n° 85), although not as much as phenolic diterpene (n° 84). There is an unassigned compound (n° 4) that is only detected in the hexane fraction although this only composed 0.6% of the mass. The compound trilobiol (n° 64), dominant in the decoction extract, was not present in the hexane fraction (Figure 41 and Figure 42).

The only compound that appears in the HC decoction and in all fractions is methyl epirosmanol isomers (n° 71 and 72), although it only appears as a trace in the DCM fraction.

The majority of different compounds present in the DCM fraction are terpenoid derivative, although the most concentrated compounds are carnosol isomers (67.2% of the mass) and rosmanol isomers (23.1% of mass) (Table 48). The carnosol isomers (n° 81 and n° 82) were enriched by the DCM fractionation and were not detected in other fractions. Another interesting observation about this fraction is that the compound salviregnone (n° 90) was identified in this sample but not in the decoction extract, this could indicate that this compound was just a trace and was enriched with the fractionation.

The methanol fraction contained a wide variety of compounds present in the original decoction extract. The most prevalent compounds were: Rosmanol and its isomers (n° 52 and 53; 32.1% of mass), trilobiol (n° 65; 23% of mass), methyl epirosmanol (n° 71, 72, 74 and 75; 11.8% of mass), isoschaftoside (n° 10; 5.9% of mass). The greatest enrichment occurred for one of the rosmanol peaks (n° 53; 12.3 x enrichment over the decoction) and a trace compound clemomandshuricoside B (n° 38) which only comprised 0.4% of the mass but was 6.9 times enriched over the decoction.

For the water fraction the most prevalent compounds were: trilobiol (n° 64; 29.7% of mass), the flavonoid glycoside, vicenin-2 (n° 6; 27.7% of mass) and shaftoside/isoschaftoside (n° 10 and 11; 14.4% of mass). The greatest enrichment was observed with vicenin-2 which was 8.5 x enriched over the decoction.

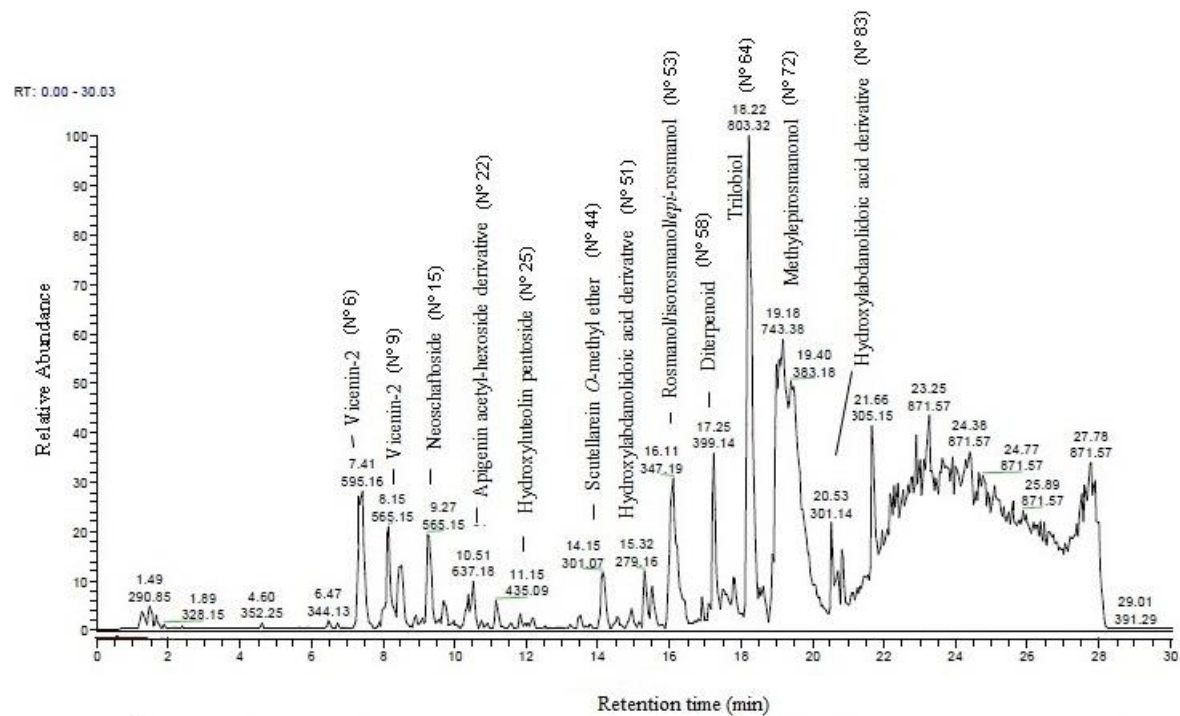


Figure 41 - HPLC-MS chromatogram of decoction extract. Chromatogram showing relative abundance, retention time and mass-to-charge (m/z) of compounds

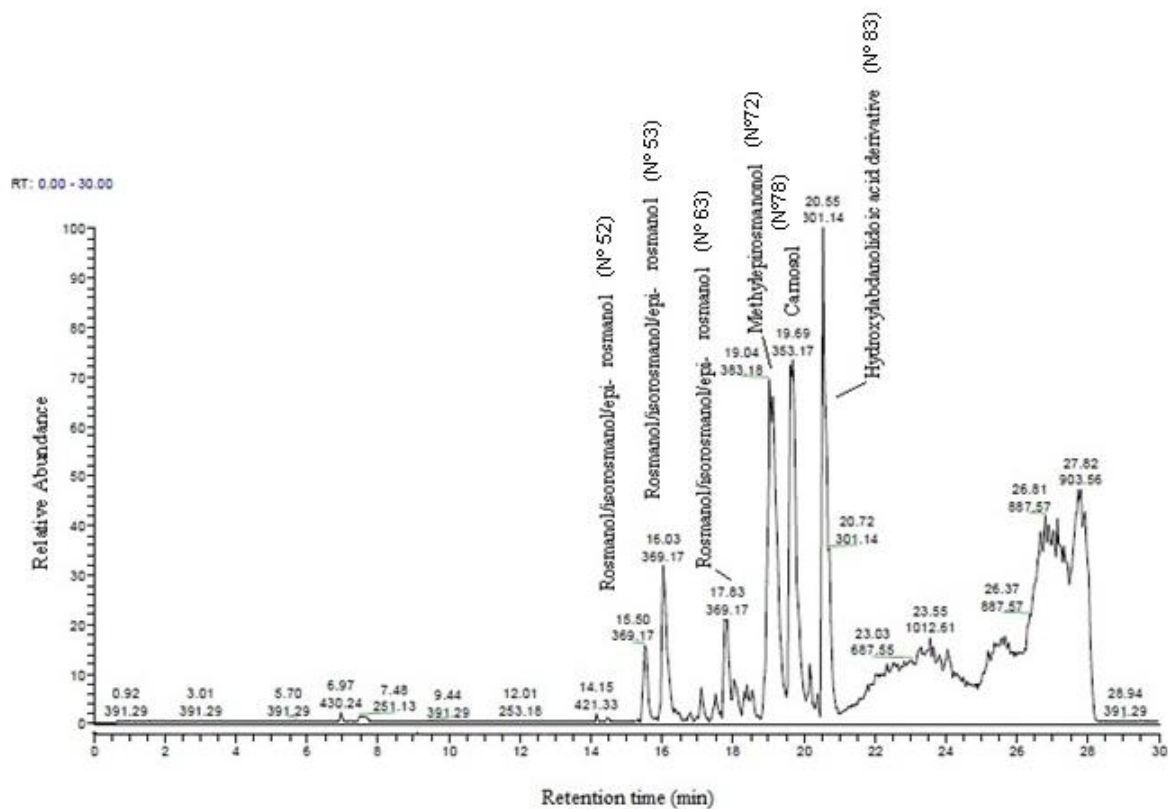


Figure 42 - HPLC-MS chromatogram of HC hexane fraction. Chromatogram showing relative abundance, retention time and mass-to-charge (m/z) of compounds

Compound number	Retention time	Compound name	DecExt area mm ²	HexFrac area mm ²	DCMFrac area mm ²	MetFrac area mm ²	WatFrac area mm ²	DecExt Amount % weight	HexFrac Amount % weight	DCMFrac Amount % weight	MetFrac Amount % weight	WatFrac Amount % weight	DecExt Conc. (45 mg)	HexFrac Conc. (2.1 mg)	DCMFrac Conc. (4 mg)	MetFrac Conc. (14.8 mg)	WatFrac Conc. (23 mg)	HexFrac Enrichment	DCMFrac Enrichment	MetFrac Enrichment	WatFrac Enrichment
1	6.5	Coumaroyl hexoside	2.0	ND	ND	0.5	ND	0.2	0.0	0.0	0.0	0.0	0.074	0.000	0.000	0.000	0.000	0.0	0.0	0.2	0.0
2	6.7	Creoside I (or isomer)	1.0	ND	ND	0.5	ND	0.1	0.0	0.0	0.0	0.0	0.035	0.000	0.000	0.000	0.000	0.0	0.0	0.3	0.0
3	6.9	Unassigned compound	0.5	ND	1.5	0.5	ND	0.0	0.0	0.1	0.0	0.0	0.013	0.000	0.004	0.000	0.000	0.0	3.4	0.7	0.0
4	7	Unassigned compound	ND	3.1	ND	ND	ND	0.0	0.6	0.0	0.0	0.0	0.000	0.013	0.000	0.000	0.000	ND	NC	ND	ND
5	7.2	Unassigned compound	0.5	ND	2.5	ND	ND	0.0	0.0	0.2	0.0	0.0	0.013	0.000	0.006	0.000	0.000	0.0	5.6	0.0	0.0
6	7.4	Vicenin-2 (or isomer)	21.8	ND	ND	9.0	49.0	3.3	0.0	0.0	4.7	27.7	1.468	0.000	0.000	0.693	6.365	0.0	0.0	1.4	8.5
7	7.5	Unassigned compound	21.8	3.0	3.5	9.0	ND	1.3	0.3	0.2	1.8	0.0	0.563	0.007	0.009	0.266	0.000	0.3	0.2	1.4	0.0
8	7.9	Benzyl disaccharide	0.5	ND	ND	0.5	ND	0.1	0.0	0.0	0.2	0.0	0.023	0.000	0.000	0.026	0.000	0.0	0.0	3.5	0.0
9	8	Vicenin-2 (or isomer)	0.5	ND	ND	0.5	ND	0.1	0.0	0.0	0.3	0.0	0.034	0.000	0.000	0.038	0.000	0.0	0.0	3.5	0.0
10	8.2	Isoschaftoside	31.5	ND	ND	12.0	13.0	4.5	0.0	0.0	5.9	7.0	2.018	0.000	0.000	0.877	1.603	0.0	0.0	1.3	1.6
11	8.5	Schaftoside	13.0	ND	ND	5.0	13.8	1.9	0.0	0.0	2.5	7.4	0.833	0.000	0.000	0.365	1.696	0.0	0.0	1.3	4.0
12	8.9	Unassigned compound	1.1	ND	0.5	ND	ND	0.1	0.0	0.0	0.0	0.0	0.037	0.000	0.002	0.000	0.000	0.0	0.5	0.0	0.0
13	8.9	Benzyl pentoside derivative	1.1	ND	ND	0.5	1.5	0.1	0.0	0.0	0.1	0.3	0.031	0.000	0.000	0.016	0.079	0.0	0.0	1.5	5.0
14	9.1	Neoisoschaftoside	0.5	ND	ND	0.5	0.5	0.1	0.0	0.0	0.2	0.3	0.032	0.000	0.000	0.037	0.062	0.0	0.0	3.5	3.8
15	9.3	Neoschaftoside	20.0	ND	ND	8.0	11.0	2.8	0.0	0.0	3.9	5.9	1.281	0.000	0.000	0.585	1.357	0.0	0.0	1.4	2.1
16	9.6	Apigenin methyl ether pentosyl hexoside	0.5	ND	ND	ND	0.5	0.1	0.0	0.0	0.0	0.3	0.033	0.000	0.000	0.000	0.063	0.0	0.0	0.0	3.8
17	9.7	Vitexin	6.0	ND	ND	0.5	1.5	0.7	0.0	0.0	0.2	0.6	0.294	0.000	0.000	0.028	0.142	0.0	0.0	0.3	0.9
18	10	Isovitexin	2.5	ND	ND	ND	0.5	0.3	0.0	0.0	0.0	0.2	0.123	0.000	0.000	0.000	0.047	0.0	0.0	0.0	0.8
19	10.3	Luteolin glycoside derivative	2.5	ND	ND	0.5	0.5	0.4	0.0	0.0	0.3	0.3	0.173	0.000	0.000	0.040	0.067	0.0	0.0	0.7	0.8
20	10.4	Sesquiterpenoid derivative	2.5	ND	0.5	0.5	ND	0.2	0.0	0.0	0.1	0.0	0.072	0.000	0.001	0.016	0.000	0.0	0.2	0.7	0.0
21	10.4	Apigenin dipentoside	2.5	ND	ND	0.5	2.0	0.3	0.0	0.0	0.2	1.0	0.152	0.000	0.000	0.035	0.234	0.0	0.0	0.7	3.0
22	10.5	Apigenin acetyl-hexoside derivative	10.0	ND	ND	4.5	4.5	1.6	0.0	0.0	2.5	2.7	0.722	0.000	0.000	0.371	0.626	0.0	0.0	1.6	1.7
23	10.7	Apigenin methyl ether pentosyl hexoside	0.5	ND	ND	0.5	0.5	0.1	0.0	0.0	0.3	0.3	0.033	0.000	0.000	0.037	0.063	0.0	0.0	3.5	3.8
24	10.9	Apigenin dipentoside	1.1	ND	ND	0.5	0.5	0.2	0.0	0.0	0.2	0.3	0.068	0.000	0.000	0.035	0.058	0.0	0.0	1.5	1.7
25	11.2	Hydroxyluteolin pentoside	6.0	ND	ND	3.0	1.9	0.7	0.0	0.0	1.1	0.8	0.296	0.000	0.000	0.169	0.178	0.0	0.0	1.7	1.2
26	11.6	Rosmarinic acid	0.5	ND	ND	0.5	0.5	0.0	0.0	0.0	0.2	0.2	0.020	0.000	0.000	0.023	0.039	0.0	0.0	3.5	3.8
27	11.6	Apigenin O-glycoside	0.5	ND	ND	ND	ND	0.1	0.0	0.0	0.0	0.0	0.023	0.000	0.000	0.000	0.000	0.0	0.0	0.0	0.0
28	11.7	Sesquiterpenoid derivative	0.6	ND	0.5	ND	ND	0.0	0.0	0.0	0.0	0.0	0.017	0.000	0.001	0.000	0.000	0.0	1.0	0.0	0.0
29	11.8	Diterpenoid derivative	0.6	ND	0.5	0.5	ND	0.0	0.0	0.0	0.1	0.0	0.022	0.000	0.002	0.021	0.000	0.0	1.0	3.0	0.0

Compound number	Retention time	Compound name	DecExt area mm ²	HexFrac area mm ²	DCMFrac area mm ²	MetFrac area mm ²	WatFrac area mm ²	DecExt Amount % weight	HexFrac Amount % weight	DCMFrac Amount % weight	MetFrac Amount % weight	WatFrac Amount % weight	DecExt Conc. (45 mg)	HexFrac Conc. (2.1 mg)	DCMFrac Conc. (4 mg)	MetFrac Conc. (14.8 mg)	WatFrac Conc. (23 mg)	HexFrac Enrichment	DCMFrac Enrichment	MetFrac Enrichment	WatFrac Enrichment
30	11.8	Megastigmenone glycoside derivative	0.6	ND	ND	0.5	0.5	0.1	0.0	0.0	0.2	0.2	0.025	0.000	0.000	0.024	0.041	0.0	0.0	3.0	3.2
31	12	Salvianolic acid B, E or L (or isomer)	0.5	ND	ND	0.5	ND	0.1	0.0	0.0	0.3	0.0	0.041	0.000	0.000	0.047	0.000	0.0	0.0	3.5	0.0
32	12.1	Megastigmenone glycoside derivative	0.5	ND	ND	0.5	ND	0.0	0.0	0.0	0.2	0.0	0.021	0.000	0.000	0.024	0.000	0.0	0.0	3.5	0.0
33	12.2	Epoxyignan derivative	1.3	ND	4.0	0.5	0.5	0.1	0.0	0.4	0.2	0.2	0.049	0.000	0.016	0.023	0.038	0.0	3.6	1.4	1.5
34	12.5	Megastigmene glycoside derivative	0.5	ND	ND	0.5	ND	0.0	0.0	0.0	0.2	0.0	0.022	0.000	0.000	0.025	0.000	0.0	0.0	3.5	0.0
35	12.6	Labdanone / kaurenone diterpenoid	0.5	ND	0.5	ND	ND	0.0	0.0	0.1	0.0	0.0	0.021	0.000	0.002	0.000	0.000	0.0	1.1	0.0	0.0
36	12.9	Sesquiterpenoid derivative	0.5	ND	ND	ND	ND	0.0	0.0	0.0	0.0	0.0	0.014	0.000	0.000	0.000	0.000	0.0	0.0	0.0	0.0
37	13.1	Labdanone / kaurenone diterpenoid	0.5	ND	ND	ND	ND	0.0	0.0	0.0	0.0	0.0	0.021	0.000	0.000	0.000	0.000	0.0	0.0	0.0	0.0
38	13.2	Clemomandshuricoside B (or isomer)	0.5	ND	ND	1.0	ND	0.1	0.0	0.0	0.4	0.0	0.029	0.000	0.000	0.065	0.000	0.0	0.0	6.9	0.0
39	13.5	Diterpenoid	2.3	ND	6.3	2.0	ND	0.2	0.0	0.6	0.6	0.0	0.081	0.000	0.022	0.082	0.000	0.0	3.1	3.1	0.0
40	13.7	5-Deacetoxy-5'-epiolguine (or isomer)	0.5	ND	ND	ND	ND	0.0	0.0	0.0	0.0	0.0	0.018	0.000	0.000	0.000	0.000	0.0	0.0	0.0	0.0
41	13.8	Megastigmadienone glycoside derivative	0.5	ND	ND	ND	ND	0.1	0.0	0.0	0.0	0.0	0.023	0.000	0.000	0.000	0.000	0.0	0.0	0.0	0.0
42	13.9	Germacradiene derivative (salviadienol B or isomer)	0.5	ND	ND	0.5	ND	0.1	0.0	0.0	0.2	0.0	0.031	0.000	0.000	0.036	0.000	0.0	0.0	3.5	0.0
43	14.1	Pervoside B (or isomer)	9.0	ND	ND	0.8	3.0	0.8	0.0	0.0	0.2	1.0	0.364	0.000	0.000	0.035	0.234	0.0	0.0	0.3	1.3
44	14.2	Scutellarein O-methyl ether	9.0	ND	8.1	3.0	ND	0.7	0.0	0.7	0.8	0.0	0.307	0.000	0.028	0.117	0.000	0.0	1.0	1.2	0.0
45	14.4	Nonyl hydroxybenzoate derivative	0.5	ND	0.5	ND	ND	0.0	0.0	0.0	0.0	0.0	0.015	0.000	0.001	0.000	0.000	0.0	1.1	0.0	0.0
46	14.5	Martiusane (or isomer)	4.5	ND	0.5	0.5	ND	0.4	0.0	0.0	0.1	0.0	0.163	0.000	0.002	0.021	0.000	0.0	0.1	0.4	0.0
47	14.7	Diterpenoid	1.4	ND	ND	0.5	ND	0.1	0.0	0.0	0.1	0.0	0.053	0.000	0.000	0.022	0.000	0.0	0.0	1.2	0.0
48	14.8	Diterpenoid	1.4	ND	ND	0.5	ND	0.1	0.0	0.0	0.2	0.0	0.059	0.000	0.000	0.024	0.000	0.0	0.0	1.2	0.0
49	14.9	Diterpenoid	1.4	ND	2.8	0.5	ND	0.1	0.0	0.3	0.1	0.0	0.053	0.000	0.010	0.022	0.000	0.0	2.2	1.2	0.0
50	14.9	Labdenone diterpenoid	1.4	ND	2.8	2.0	ND	0.1	0.0	0.3	0.6	0.0	0.056	0.000	0.011	0.091	0.000	0.0	2.2	4.9	0.0
51	15.3	Hydroxylabdanolidic acid derivative (norambreinolide-18,6-olide or isomer)	9.4	ND	ND	2.0	ND	0.7	0.0	0.0	0.5	0.0	0.296	0.000	0.000	0.072	0.000	0.0	0.0	0.7	0.0
52	15.5	Rosmanol / isorosmanol / epi-rosmanol / epi-isorosmanol	9.0	24.0	42.0	2.0	ND	0.8	3.9	4.1	0.6	0.0	0.354	0.082	0.164	0.090	0.000	5.0	5.2	0.8	0.0
53	16.1	Rosmanol / isorosmanol / epi-rosmanol / epi-isorosmanol	29.3	49.5	111.0	104.0	ND	2.6	8.1	10.9	31.5	0.0	1.153	0.170	0.434	4.663	0.000	3.2	4.2	12.3	0.0
54	16.2	Rosmanol / isorosmanol / epi-rosmanol / epi-isorosmanol	29.3	ND	ND	ND	4.5	2.6	0.0	0.0	0.0	1.5	0.000	0.000	0.000	0.000	0.000	0.0	0.0	0.0	0.6
55	16.3	Maocrystal V (or isomer)	29.3	ND	ND	ND	ND	2.4	0.0	0.0	0.0	0.0	1.153	0.000	0.000	0.000	0.341	0.0	0.0	0.0	0.0
56	16.9	Scutellarein di-O-methyl ether	0.5	ND	ND	ND	ND	0.0	0.0	0.0	0.0	0.0	1.100	0.000	0.000	0.000	0.000	0.0	0.0	0.0	0.0
57	17.1	Rosmaquinone (or isomer)	0.5	ND	ND	ND	ND	0.0	0.0	0.0	0.0	0.0	0.018	0.000	0.000	0.000	0.000	0.0	0.0	0.0	0.0
58	17.2	Diterpenoid	46.3	ND	ND	ND	10.0	4.4	0.0	0.0	0.0	3.6	0.020	0.000	0.000	0.000	0.000	0.0	0.0	0.0	0.8

Compound number	Retention time	Compound name	DecExt area mm ²	HexFrac area mm ²	DCMFrac area mm ²	MetFrac area mm ²	WatFrac area mm ²	DecExt Amount % weight	HexFrac Amount % weight	DCMFrac Amount % weight	MetFrac Amount % weight	WatFrac Amount % weight	DecExt Conc. (45 mg)	HexFrac Conc. (2.1 mg)	DCMFrac Conc. (4 mg)	MetFrac Conc. (14.8 mg)	WatFrac Conc. (23 mg)	HexFrac Enrichment	DCMFrac Enrichment	MetFrac Enrichment	WatFrac Enrichment
59	17.5	Eudesmenone or labdanolide derivative	0.5	0.5	ND	ND	ND	0.0	0.1	0.0	0.0	0.0	1.976	0.000	0.000	0.000	0.822	1.9	0.0	0.0	0.0
60	17.5	Epoxyabietrienone methyl ether derivative	0.5	ND	ND	0.5	ND	0.0	0.0	0.0	0.2	0.0	0.000	0.000	0.000	0.000	0.000	0.0	0.0	3.5	0.0
61	17.6	Diterpenoid	0.5	ND	0.5	ND	ND	0.0	0.0	0.0	0.0	0.0	0.017	0.001	0.000	0.000	0.000	0.0	1.1	0.0	0.0
62	17.6	Epoxyabietrienone methyl ether derivative	0.5	ND	ND	ND	ND	0.0	0.0	0.0	0.0	0.0	0.021	0.000	0.000	0.024	0.000	0.0	0.0	0.0	0.0
63	17.8	Rosmanol / isorosmanol / epi-rosmanol / epi-isorosmanol	11.0	22.0	52.5	ND	ND	1.0	3.6	5.1	0.0	0.0	0.019	0.000	0.002	0.000	0.000	3.7	5.3	0.0	0.0
64	18.2	Trilobiol (or isomer)	154.5	ND	ND	33.8	40.0	30.4	0.0	0.0	23.0	29.7	0.021	0.000	0.000	0.000	0.000	0.0	0.0	0.8	1.0
65	18.4	Diterpenoid	0.5	ND	0.5	ND	ND	0.0	0.0	0.0	0.0	0.0	0.432	0.076	0.205	0.000	0.000	0.0	1.1	0.0	0.0
66	18.4	Carnosic acid / 7,20:11,20-diepoxy-7,8-seco-8,11,13-abietatriene-7,14-diol / horminone (or isomer)	0.5	0.5	0.5	0.5	0.5	0.0	0.1	0.0	0.1	0.2	13.691	0.000	0.000	3.411	6.824	1.9	1.1	3.5	3.8
67	18.6	Diterpenoid	0.5	ND	33.0	ND	ND	0.0	0.0	3.1	0.0	0.0	0.019	0.000	0.002	0.000	0.000	0.0	73.9	0.0	0.0
68	18.6	Triterpenoid	0.5	ND	ND	0.5	ND	0.1	0.0	0.0	0.2	0.0	0.019	0.002	0.002	0.022	0.036	0.0	0.0	3.5	0.0
69	18.7	Methyl epirosmanol / oxoinuroyleanol (or isomer)	ND	ND	ND	ND	4.0	0.0	0.0	0.0	0.0	1.4	0.019	0.000	0.125	0.000	0.000	ND	ND	ND	NC
70	18.8	Rosmaquinone A or B (or isomer)	0.5	ND	ND	0.5	ND	0.0	0.0	0.0	0.2	0.0	0.029	0.000	0.000	0.034	0.000	0.0	0.0	3.5	0.0
71	19	Methyl epirosmanol / oxoinuroyleanol (or isomer)	61.0	90.0	0.5	16.0	6.8	5.5	15.3	0.1	5.0	2.3	0.000	0.000	0.000	0.000	0.315	2.8	0.0	0.9	0.4
72	19.1	Methyl epirosmanol / oxoinuroyleanol (or isomer)	61.0	90.0	0.5	16.0	6.8	5.5	15.3	0.1	5.0	2.3	0.020	0.000	0.000	0.023	0.000	2.8	0.0	0.9	0.4
73	19.3	Salvidorol (or isomer)	0.5	ND	17.0	ND	ND	0.0	0.0	1.5	0.0	0.0	2.495	0.322	0.002	0.746	0.532	0.0	38.1	0.0	0.0
74	19.4	Methyl epirosmanol / oxoinuroyleanol (or isomer)	34.7	ND	ND	3.0	ND	3.2	0.0	0.0	0.9	0.0	2.495	0.322	0.002	0.746	0.532	0.0	0.0	0.3	0.0
75	19.5	Methyl epirosmanol / oxoinuroyleanol (or isomer)	34.7	ND	ND	3.0	ND	3.2	0.0	0.0	0.9	0.0	0.018	0.000	0.061	0.000	0.000	0.0	0.0	0.3	0.0
76	19.6	Carnosol / hyptol (or isomer)	34.7	76.0	ND	ND	ND	2.9	11.9	0.0	0.0	0.0	1.418	0.000	0.000	0.140	0.000	4.1	0.0	0.0	0.0
77	19.6	Epoxykaurene derivative	34.7	ND	ND	3.0	ND	3.6	0.0	0.0	1.1	0.0	1.418	0.000	0.000	0.140	0.000	0.0	0.0	0.3	0.0
78	19.7	Carnosol / hyptol (or isomer)	34.7	76.0	180.3	ND	ND	2.9	11.9	16.8	0.0	0.0	1.300	0.249	0.000	0.000	0.000	4.1	5.8	0.0	0.0
79	19.8	Abietadi- or tri-ene derivative	34.7	ND	ND	3.0	ND	3.4	0.0	0.0	1.0	0.0	1.599	0.000	0.000	0.158	0.000	0.0	0.0	0.3	0.0
80	19.9	Methoxynepetaeofolin derivative (or isomer)	0.5	ND	ND	0.5	ND	0.1	0.0	0.0	0.2	0.0	1.300	0.249	0.673	0.000	0.000	0.0	0.0	3.5	0.0
81	20	Carnosol / hyptol (or isomer)	0.5	ND	180.3	ND	ND	0.0	0.0	16.8	0.0	0.0	1.536	0.000	0.000	0.152	0.000	0.0	403.8	0.0	0.0
82	20.1	Carnosol / hyptol	0.5	ND	180.3	ND	ND	0.0	0.0	16.8	0.0	0.0	0.025	0.000	0.000	0.028	0.000	0.0	403.8	0.0	0.0
83	20.5	Hydroxylabdanolidic acid derivative	11.5	68.7	ND	ND	ND	0.8	9.0	0.0	0.0	0.0	0.019	0.000	0.673	0.000	0.000	11.2	0.0	0.0	0.0
84	20.6	Carnosol / hyptol (or isomer)	0.5	68.7	180.3	ND	ND	0.0	10.7	16.8	0.0	0.0	0.019	0.000	0.673	0.000	0.000	257.2	403.8	0.0	0.0
85	20.7	Hydroxylabdanolidic acid derivative	9.0	68.7	ND	ND	10.0	0.6	9.0	0.0	0.0	2.6	0.363	0.190	0.000	0.000	0.000	14.3	0.0	0.0	4.2
86	20.8	Methyl pisiferate (or isomer)	0.5	0.5	ND	ND	ND	0.0	0.1	0.0	0.0	0.0	0.019	0.225	0.673	0.000	0.000	1.9	0.0	0.0	0.0

Compound number	Retention time	Compound name	DecExt area mm ²	HexFrac area mm ²	DCMFrac area mm ²	MetFrac area mm ²	WatFrac area mm ²	DecExt Amount % weight	HexFrac Amount % weight	DCMFrac Amount % weight	MetFrac Amount % weight	WatFrac Amount % weight	DecExt Conc. (45 mg)	HexFrac Conc. (2.1 mg)	DCMFrac Conc. (4 mg)	MetFrac Conc. (14.8 mg)	WatFrac Conc. (23 mg)	HexFrac Enrichment	DCMFrac Enrichment	MetFrac Enrichment	WatFrac Enrichment
87	20.8	Arucadiol / hydroxy-miltirone (or isomer)	8.5	ND	ND	ND	ND	0.6	0.0	0.0	0.0	0.0	0.284	0.190	0.000	0.000	0.608	0.0	0.0	0.0	0.0
88	21.5	Ethylrosmanol / epi-ethylrosmanol (or isomer)	0.5	ND	28.0	ND	ND	0.0	0.0	3.0	0.0	0.0	0.019	0.002	0.000	0.000	0.000	0.0	62.7	0.0	0.0
89	21.7	Abietane diterpenoid derivative	0.5	0.5	0.5	ND	ND	0.0	0.1	0.0	0.0	0.0	0.288	0.000	0.000	0.000	0.000	1.9	1.1	0.0	0.0
90	22.1	Salviregnone (or isomer)	ND	ND	21.8	ND	ND	0.0	0.0	1.7	0.0	0.0	0.021	0.000	0.118	0.000	0.000	ND	ND	NC	ND
91	22.8	Esquirolin B / suaveololi (or isomer)	0.5	0.5	0.5	0.5	ND	0.0	0.1	0.0	0.1	0.0	0.000	0.000	0.000	0.000	0.000	1.9	1.1	3.5	0.0

Table 48 - Concentration of compounds present in the *H. crenata* decoction extract and enrichment of its fractions (hexane, DCM, methanol and water). DecExt= Decoction extract, HexFrac= Hexane fraction, DCMFrac=DCM fraction, MetFrac=Methanol fraction, WatFrac=Water fraction. ND= Compound not detected, NC= Not calculated due not detection of compound in the decoction extraction. Area mm² = Measurement of compound peak's area on the HPLC-MS chromatogram. Amount % weight = Percentage of compound amount according to the total mass of compounds in the sample. Conc. = Amount of *H. crenata*'s dry matter in the sample. Enrichement = Proportion of enrichment of the fraction over decoction extract. Some of the compounds presented in this table, such as N^o 53, 54, 71 and 72 have the same molecular formula and molecular weight, however they could not be distinguished because no reference compounds were available to compare them with (the retention times and MS data). For this reason these compounds appear more than once in the table.

7.4 Discussion

This study investigated which compounds are present in the *H. crenata* preparations tested. The results have shown the presence of 91 compounds, some of which were also reported in other *Hyptis* species. These compounds are apigenin, carnosic acid, carnosol, esquirolin B, hyptol, martiusane, methoxynepetefolin derivative, rosmarinic acid and salvigenin, as shown in the *Hyptis* genus compounds table in Appendix A. Therefore, through the results of this project 82 compounds were described that are unknown to the knowledge of the *Hyptis* genus chemical profile.

The compounds present in the hexane fraction will be the main focus of this discussion since this fraction was the most potent treatment tested. There were 15 compounds enriched by this fractionation. The highest enrichment observed was for carnosol/hyptol followed by hydroxyabdanolidoic acid and rosmanol. However if compared with other fractions tested, which were less active than the decoction itself, there are 7 compounds that are more enriched by hexane fractionation. These compounds are eudesmenone (n° 59), methyl epirosmanol (n° 71 and n° 72), carnosol/hyptol (n° 76), hydroxyabdanolidoic acid derivative (n° 83 and n° 85) and methyl pisiferate (n° 86). With the exception of compounds 71, 72 and 85, the other compounds are only present in the hexane fraction. It is possible that one compound, some compounds or the entire hexane composition could be contributing to the antinociceptive effect observed, in particular those selectively enriched by the hexane fractionation. Therefore, the next step of this study should be to isolate these compounds and test them separately, using the hexane fraction as a positive control. The results might indicate if any of these isolated compounds would be more potent than the hexane fraction. If the fraction were to be the most potent, then it could be proposed that compounds are enhancing each other's effects, therefore it could be a synergistic activity.

The fact that in the thermal antinociceptive test the water fraction was significantly effective, but the methanol fraction was not, could eliminate the possibility that compounds 71 and 72 were the ones responsible for the antinociceptive effect, since their concentration in the methanol fraction is higher than in the water fraction. Therefore, it could be suggested that the effect observed from the water fraction could be induced by other compounds such as vicenin-2 (n° 6), which had a high enrichment within the water fraction. Besides, vicenin-2 has been already reported for this effect (Gorzalczany *et al.*, 2011). Another potential compound that might be inducing the

effect observed from both fractions is hydroxylabdanolidoic acid derivative (n° 83), since it is present in both fractions but with higher concentration in the hexane fraction.

Among these compounds described in the hexane fraction, carnosol derivative followed by rosmanol derivative are the most mentioned compounds in the literature. The compound carnosol was first observed in the species *Rosmarinus officinalis* L as well as the compound rosmanol (Wenkert *et al.*, 1965; Nakatani and Inatani, 1981). After these reports, carnosol has been described in several species, some of these species are *Salvia officinalis*, *Origanum majorana*, *Origanum vulgare*, *Dorystoechas hastate* and *Sphacele chamaedryoides* (Abreu *et al.*, 2008; Areche *et al.*, 2009b; Farhat *et al.*, 2009; Erkan *et al.*, 2011; Bauer *et al.*, 2012; Cui *et al.*, 2012; Hossain *et al.*, 2012; Walch *et al.*, 2012). The compound rosmanol, which is a abietane diterpene, is also commonly found in the *Salvia* species (Djarmati *et al.*, 1992; Miura *et al.*, 2001; Guerrero *et al.*, 2006; Máthé *et al.*, 2007; Cui *et al.*, 2012). These two compounds are also mentioned in the literature for their pharmacological activities as antidepressant, gastroprotective, neuroprotective and anti-inflammatory effects (Izumi *et al.*, 2007; Areche *et al.*, 2009a; Beninca *et al.*, 2011). However only carnosol is reported for antinociceptive activities (Rodrigues *et al.*, 2012).

As shown in Table 48, compound n° 76 may be a hyptol rather than a carnosol derivative. Hyptol is not widely known in relation to its pharmacology, so far there is no report about it. However its structure is described from chemical studies with the species *H. fruticosa* (Monache *et al.*, 1977).

Hydroxylabdanolidoic acid derivative, one of the most enriched compounds in the hexane fraction, as well as the other less enriched compounds methyl pisiferate (n° 86) and eudesmenone (n° 59), are also not widely known. Hydroxylabdanolidoic acid was reported for the Lamiaceae subfamilie nepetoideae (CCD, 2012), the name labdane comes from the Mediterranean shrub *Cistus ladaniferus* which secretes a dark brown resin containing α -pinene and 8β hydroxyl labdan -15-oic acid (Breitmaier, 2006). Labdanoic acid is found in high concentrations in the leaves of *Aristolochia giberti* which is widely used in Brazilian tradition medicine (Marchesini *et al.*, 2009). Methyl pisiferate had been reported as a diterpene from the *Chamaecyparis pisifera* species (Yatagai and Takahashi, 1979), whereas eudesmenone compounds have only been reported as components of vetiver oil.

7.5 Conclusions

Based on the enrichment calculation for the hexane fractionation, it can be concluded that there are possible compounds that might play an important role in the antinociceptive effect observed. These compounds are hydroxyabdanolidoic acid, methyl epirosmanol, carnosol or hyptol and methyl piperate derivative.

**Chapter 8. General Discussion, concluding remarks and
recommendations**

8.1 General discussion

8.1.1 Medical plants, past and future

Plants and man, through the process of evolving, have always kept a close relationship. There are many ways that man uses the benefits offered by plants and one of them is the medicinal properties obtained from metabolites of defence against environmental conditions produced in the plant. Over the years mankind learned about which species could be used and their activities, therefore there are thousands of medicinal plants that are traditionally used and thousands of different uses. Because the effects obtained could not be reasonably explained, throughout history they have been associated with magic, god beliefs and mysticism. However, with the evolution of medicine and chemistry the medicinal properties of plants are finally being properly explained. This started with the discovery of morphine in 1804, unveiling the main key behind the effect of *Papaver somniferum* (Klockgether-Radke, 2002). After this event, the interest grew in screening medicinal plants for therapeutic compounds, however the aim was primarily towards compound synthesis (Borris, 1996).

Therefore, this era of synthesis left a legacy of drugs in the market for which the active compound is based on a medicinal plant substance. However, in more recent years, there has been a new focus on the use and commercialisation of medicinal plants in the form of developing phytotherapeutics and perhaps this is the future for the use of medicinal plants as medication (Desmarchelier, 2008). A phytotherapeutic product is the result of preparation of a medicinal plant using standard methods and controlling the quality and quantity of compounds in order to deliver safer and more efficient plant medication (Calixto, 2000). The use of phytotherapeutics and nutraceuticals, which is also part of herbal therapy, has been widely accepted in the United States, Europe and Asia (Iriti *et al.*, 2010). In Brazil, since 2008, the Brazilian National Health System (Sistema Unico de Saude -SUS) has included phytotherapeutics as part of their list of medication to be distributed in sixteen federal states, which contains six medicinal plants for producing these phytotherapeutics (SUS, 2010). However, the number of plants included on the list is very small when compared to the diversity of medicinal plants found in Brazil (da Cunha and Bortolotto, 2011). The main reason why there is not a larger number of medicinal plants that can be used as phytotherapeutics is due to a lack of pharmacological information about the species. One such species is *Hyptis crenata* Pohl.

8.1.2 *The traditional use survey and its contribution to this study*

Species from the *Hyptis* genus have been in traditional use for generations and some *Hyptis* species have shown pharmacological activities in pre-clinical trials. However, there has been no pharmacological study of *H. crenata*, and the fact that it was used traditionally as medicine could indicate potential therapeutic activities. Considering the benefits of knowing details about its use and method of preparation, before carrying out pharmacological trials, the first step of this project was to carry out a small traditional use survey. The results showed that this plant is mainly used for the relief of pain and there are seven different ways to prepare the plant, with each method extracting a different concentration and type of compounds, in this case volatile and phenolic compounds. Despite there being a previous survey about the traditional use of *H. crenata* published during the course of this thesis (De Jesus *et al.*, 2009), the researchers only reported one method of preparation and without details of its procedure. Therefore, this thesis is the first to suggest that temperature and solvent, which varied according to the different method of preparation, have an important role in determining the amount and type of *H. crenata* compounds obtained.

The next step of this project was to choose which of these extracts would be suitable to test for its antinociceptive properties. Although it could be interesting to investigate whether all these different methods would induce antinociception, in order to minimise the number of animals used and because it was a proof of principle investigation, it was decided to test the method of preparation that was the most recommended by the interviewees. For this study it was decided not to test the volatiles due to their low level in the decoction when compared with other extraction methods mentioned in the traditional use survey. This could indicate a preference by the users for the lowest amount of this type of compound in the preparation. This preference could suggest that these compounds are not important for the effect or there is a risk of toxicity related to them. This theory may be more likely when the reports of poisoning caused by camphor, which was one of the main volatiles found in these different preparations, are taken into consideration (Manoguerra *et al.*, 2006; Bhaya and Beniwal, 2007a). Although volatile compounds in *Hyptis* have been demonstrated to induce antinociception (Menezes *et al.*, 2007; Arrigoni-Blank *et al.*, 2008; Franco *et al.*, 2011; Raymundo *et al.*, 2011), the data presented in this thesis clearly demonstrated that *H. crenata* treatment not containing

volatile compounds efficiently induced antinociception from thermal and chemical noxious stimuli.

8.1.3 *The antinociceptive effect of *H. crenata* and possible anxiolytic effect*

This study showed that *H. crenata* extract dose-dependently increased the threshold for peripheral thermal stimuli and visceral chemical stimuli, but it is still unclear where the site of action for the active compounds is. However, the fact that *H. crenata* was shown to be effective in both of these animal models could suggest that its treatment can inhibit pain transmitted through A δ and C fibres, as A δ is especially involved in the thermal pain response measured in the Hargreaves test and C fibres are the main pain connection for the animal model of peritoneo-visceral chemical stimuli (Calvino *et al.*, 1984; Le Bars *et al.*, 2001). Because C fibres are also the main fibres involved in chronic pain sensation (Ørstavik *et al.*, 2003), for future studies it would be interesting to evaluate *H. crenata* treatment for chronic pain symptoms. Indeed, the fact that this plant treatment dose-dependently decreased c-fos in the hypothalamus after acetic acid stimulus would make this prospect for a chronic pain trial even more attractive, since this decrease in neurone activity in the hypothalamus suggests that *H. crenata* effectively reduced the stress caused by the pain sensation (Galli *et al.*, 2009). Another recommendation for the future evaluation of *H. crenata* would be to investigate its effect against headache like migraine, since the hypothalamus plays an important role in the mechanism for this symptom (Matharu, 2007). In addition, the fact that headache was one of the symptoms that are treated with *H. crenata* reported in the traditional use survey further supports this proposal.

The fact that only the higher dose tested (HC 150 mg/kg (p.o.)) significantly reduced c-fos activity could indicate that the compound(s) responsible for this effect has a lower concentration compared to the others that compose the chemical profile of *H. crenata*. Because anxiety stimuli increase c-fos in the PVN (see Chapter 5), while anxiolytic compounds decrease this expression (Zahner *et al.*, 2007), this compound may be decreasing c-fos through an anxiolytic effect, since the antinociceptive action in the acetic acid test HC 15 mg/kg (p.o.) was as effective as the higher dose, therefore in theory both should have the same effect if only pain was influencing the c-fos level. The compound that was observed in *H. crenata* samples, and that would most likely fit these descriptions is the derivative of flavonoid shaftoside, which is also found in the species *Passiflora incarnata* L and has been described as an anxiolytic and sedative (Wohlmuth

et al., 2010). A simple approach for future study to find out if this compound or other compounds is/are responsible for the c-fos activity decrease, would be to analyse the brain of animals treated with *H. crenata* fractions after performing the acetic acid test. If the effect only appears for the methanol and water fractions, and the effect of the water fraction is stronger than that of the methanol fraction, it is likely that shaftoside derivative could be the compound involved, since this compound is more concentrated in the water fraction. Although shaftoside derivative has been reported as a sedative, it seems that it is not having this effect according to the behavioural results presented in this thesis from animals treated with the *H. crenata* extract. However, the anxiolytic effect of the extract was not analysed, therefore from these results it is not possible to affirm if this plant does or does not induce this activity, although it is possible to propose a likelihood based on the c-fos reduction induced by the extract and supported by the fact that there is another species of *Hyptis* that was confirmed to have this effect in a pre-clinical trial (Bueno *et al.*, 2006).

8.1.4 Possible *H. crenata* compounds involved in the antinociceptive effect

The results in this thesis showed that in both animal models for thermal and visceral nociception the most active *H. crenata* treatment was the hexane fraction. Interestingly, this fraction was the one with the smallest amount of dry material (2.1 mg/kg (p.o.)), and this suggests that the compounds that constitute the hexane fraction are more involved in the mechanism of pain relief. The compounds that have the highest enrichment in the hexane fraction were carnosol (n° 84), hydroxylabdanolidoic acid (n° 85) and rosmanol (n° 52).

Previous work evaluating rosmanol derivatives suggested that it performs an important role as an anti-inflammatory agent by inhibiting the activity of transcription factors NF- κ B and STAT3 that play a key role in regulating COX-2 and inducible nitric oxide synthase (iNOS) gene expression. It was also proposed that this inhibition is through suppression of phosphorylation and degradation of I κ B, which is part of the NF- κ B transduction cascade (Lai *et al.*, 2009). The enzyme COX-2 catalyses prostaglandin E2 production and iNOS synthesises nitric oxide, both molecules are pro-inflammatory and pain mediators (Koch *et al.*, 2007; Tassorelli *et al.*, 2007). Hence, these reports imply that the antinociceptive effect observed from *H. crenata* treatment could involve inhibition of both these enzymes, but mainly COX-2 since its activity increases during the acetic acid test. In addition, carnosol also has been reported as a COX-2 inhibitor.

The mechanism of COX-2 inhibition by carnosol has been clearly elucidated in the literature. This compound inhibits microsomal prostaglandin E Synthase-1 (mPGES-1), which is a COX-2 promoter. This process is achieved by interruption of the protein kinase C signalling which consequently affects the binding of the activator protein-1 (AP-1) to the cyclic adenosine monophosphate response for mPGES-1 (Subbaramaiah *et al.*, 2002; Samuelsson *et al.*, 2007; Bauer *et al.*, 2012). However, when considering the fact that the concentrations of carnosol (n° 84) and rosmanol derivatives (n° 52) were higher in the DCM fraction sample than the hexane fraction and the fact that thermal and visceral antinociception evaluations showed that the DCM fraction was much less active than the hexane fraction, it could indicate that although these compounds might be having some effect, they are not the most important compounds in the sample responsible for the antinociceptive effect observed. On the other hand, it is likely that this role might be played by eudesmenone (n° 59), methyl epirosmanol (n° 71 and n° 72), carnosol/hyptol isomer (n° 76), hydroxylabdanolidoic acid derivative (n° 83 and n° 85) and methyl pisiferate (n° 86), since the concentrations of these compounds are higher in the hexane fraction when compared to the DCM fraction.

Searches of the literature were made to identify whether there were biological activities reported for these compounds. There are reports of methyl epirosmanol identified in other *Hyptis* species (Urones *et al.*, 1998; Araujo *et al.*, 2005), as well as a report of an antioxidant and anti-inflammatory study from *Salvia namaensis* containing this compound (Kamatou *et al.*, 2010). However, there were no reports about the biological activities of the other compounds. Regarding the *Salvia namaensis* anti-inflammatory study, in contrast to rosmanol as discussed earlier, the treatment containing methyl epirosmanol was ineffective against inflammation. Therefore, it could indicate that this compound might be a specific antinociceptive agent.

In hypothesis that these compounds might be the ones responsible for the antinociceptive effect and that the concentration of compound enrichment might play an important role in the antinociceptive effect, the enrichment of these compounds in the hexane, methanol and water fractions were compared. As the hexane fraction was the most potent in the Hargreaves test, and the water fraction was also effective, it could be expected that concentration of the active compounds should be highest in the hexane fraction, less high in the water fraction and lowest in the methanol fraction. Therefore, the only compound that would fit this profile is hydroxylabdanolidoic acid (n° 85).

On the other hand, it is also possible that the effect induced by the water fraction could be induced by a compound not present in the hexane fraction. In this case, the most likely compounds inducing the antinociceptive effect observed in the hexane fraction would be compounds n° 59, n° 71, n° 72, n° 76, n° 83, n° 85 and n° 86.

However, there is another possibility that involves the potential of carnosol (which had the highest concentration in the hexane fraction and has been reported as having antinociceptive effect) being the active compound if considering the fact that the hexane fraction had fewer compounds than the other inactive fractions. It is possible that the fact that the DCM fraction also had this compound in such a high concentration, but not the effect, could be due to another compound that is inhibiting or antagonizing the action of carnosol (Rowland and Tozer, 2011). Therefore, perhaps other compounds present in hexane fraction are not playing an important role as antinociceptive, they are just being inactive, allowing rosmanol and carnosol to induce their effect. Alternatively it could be that a number of compounds in the hexane fraction are active, but because they are pain specialised they would work in a synergetic way amplifying the antinociception effect that was slightly observed in the water fraction.

It is also possible that the effect observed in the Hargreaves test and in the acetic acid test could be obtained by two different mechanisms that were induced by different compounds. Carnosol and rosmanol for example, could be inhibiting PGE 2 in the peritoneal fluid in the acetic acid test and the other compounds could be acting to inhibit the withdrawal reflex promoted by A δ fibres in the Hargreaves test. A future methodological consideration would be to measure the level of prostaglandin in the peritoneal fluid of mice treated with the hexane fraction and its isolated compounds after injection of acetic acid. In addition it would be recommended to evaluate these treatments together with naloxone to examine if this opioid antagonist would reverse the antinociceptive effects (Chahl, 1996; Dawood and Khan-Dawood, 2007). An opioid effect was suggested for other *Hyptis* species using this approach (Bispo *et al.*, 2001; Santos *et al.*, 2007b), although it was not shown which *Hyptis* compounds might be inducing this effect.

8.1.5 Possible anti-inflammatory effect of *H. crenata*

When searching in the literature for antinociceptive and anti-inflammatory effects reported for the compounds presented in the *H. crenata* treatments, it was found that

there are eleven compounds (vicenin-2, apigenin, vitexin, luteolin, rosmarinic acid, salvianolic acid, scutellarein, rosmanol, carnosic acid, suaveolol and carnosol) that have been reported as anti-inflammatory agents (Hu and Kitts, 2004; Van Dross *et al.*, 2005; Chen *et al.*, 2006b; Scheckel *et al.*, 2008; Lai *et al.*, 2009; Marrassini *et al.*, 2011). The fact that 12 % of *H. crenata* compounds are reported to have anti-inflammatory effects indicates the possibility for *H. crenata* treatment to have this activity. In addition, carnosic acid and suaveolol have already been reported as responsible for the anti-inflammatory effect observed in *H. verticillata* and *H. brevipes* (Shenoy and Shirwaikar, 2002; Grassi *et al.*, 2006; Raederstorff *et al.*, 2008). For this reason, it would be recommended to investigate *H. crenata* treatment in an animal model to evaluate its anti-inflammatory effect.

8.1.6 Contribution of this thesis to the chemistry of *Hyptis* genus

So far, the majority of chemical compounds reported for the *Hyptis* genus, and especially for *H. crenata*, are volatile compounds obtained from essential oils. One of the motives for mainly studying volatile compounds is due to the fact that they are relatively easy to be identified, since the equipment most commonly used is GC-MS which is more accessible than HPLC-MS. For this reason, 82 compounds reported in this study were not available in the literature for *Hyptis* species. Therefore, knowledge of the chemistry of this genus has been improved.

8.2 Concluding remarks and recommendations

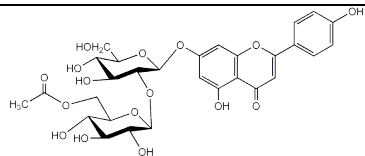
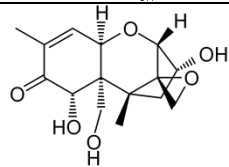
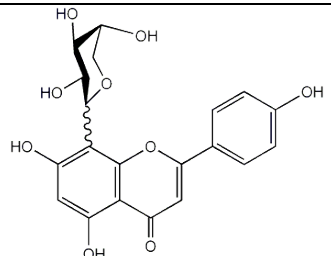
As the current pain relief treatments available in the market are still failing to treat some pain symptoms (Jensen and Finnerup, 2007), and the fact that people are looking for more “natural” treatments for symptoms like menstrual pain which is suffered by 50% of worldwide female population (Dawood, 2006), it might be recommended to evaluate *H. crenata* as a possible phytotherapeutic in a clinical trial for this symptom, since it was effective against visceral pain and it has indicated to inhibit prostaglandin E2, which is one of the pain mediators amplified during this symptom (Hayes and Rock, 2002).

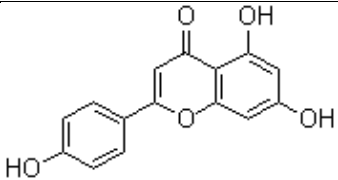
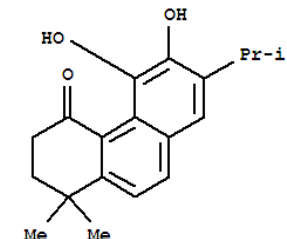
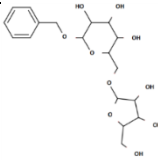
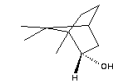
If *H. crenata* would be evaluated as a possible phytotherapeutic in a clinical trial, firstly it would be recommended to carry out a detailed toxicology analysis of the plant. Secondly, the treatment to be evaluated should be the hexane fraction for its potency or the aqueous decoction extract, prepared at a concentration of 50g/l, heating it until it

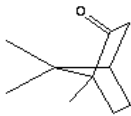
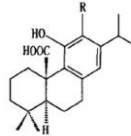
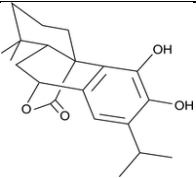
boiled (5 min boiling). In addition, the compounds hydroxyabdanolidoic acid derivative, methyl epirosmanol isomer, rosmanol isomer, eudesmenone derivative, methyl pisiferate and carnosol isomer present in the hexane fraction are the possible compounds to be used as markers for the extract standardisation.

Taken together, *H. crenata* has potential as a pain relief treatment and also as a potential anti-inflammatory treatment. Its effect and mechanism should be further investigated in order to find out its site of action in the pain and anti-inflammatory pathways and to investigate which specific compounds are responsible for this effect. Therefore for future studies it would be recommended to test each of those compounds that are in the hexane fraction and evaluate their activities.

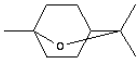
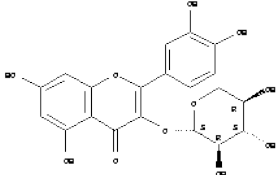
Appendix A – *Hyptis crenata* Chemical Compounds

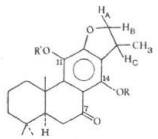
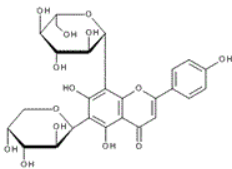
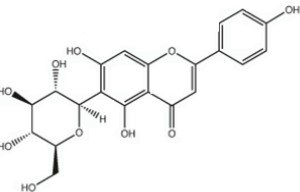
Compound	Molecular formula	Structure	Molecular weight/ (g/mol)	<i>Hyptis</i> Species/Author	Pharmacological effects Applied for the compound published.
Abietadi- or tri-ene derivative	C ₂₂ H ₃₀ O ₆	Not found	390.1936	<i>H. crenata</i> (present work)	Anti-inflammatory activities(Pferschy-Wenzig <i>et al.</i> , 2008)
Abietane diterpenoid derivative	C ₂₁ H ₂₈ O ₃	Not found	328.193	<i>H. crenata</i> (present work)	Not described ^A
Apigenin acetyl-hexoside derivative	C ₂₉ H ₃₂ O ₁₆	 The structure shows a flavone core (apigenin) with a 4'-hydroxyphenyl group at the 7-position. It is linked via an ether bond to a hexose sugar, which is further substituted with an acetyl group (H ₃ C-CO-) and a hydroxymethyl group (HOH ₂ C-).	636.1761	<i>H. crenata</i> (present work)	Not mentioned
Apigenin dipentoside	C ₂₅ H ₂₆ O ₁₃	 The structure shows a complex diterpenoid skeleton with multiple hydroxyl groups and a lactone ring, representing a dipentoside derivative of apigenin.	534.1445	<i>H. crenata</i> (present work)	Not mentioned
Apigenin methyl ether pentosyl hexoside	C ₂₇ H ₃₀ O ₁₄	Not found	578.1713	<i>H. crenata</i> (present work)	Not mentioned
Apigenin <i>O</i> -glycoside	C ₂₀ H ₁₈ O ₉	 The structure shows the apigenin core with a 4'-hydroxyphenyl group at the 7-position. It is linked via an ether bond to a pentose sugar, which is further substituted with a hydroxyl group (HO-).	402.1024	<i>H. crenata</i> (present work)	Not mentioned

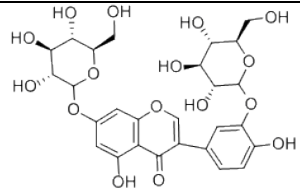
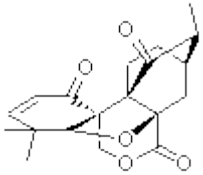
Compound	Molecular formula	Structure	Molecular weight/ (g/mol)	<i>Hyptis</i> Species/Author	Pharmacological effects Applied for the compound published.
Apigenin	C ₁₅ H ₁₀ O ₅		270.2	<i>H. albida</i> <i>H. capitata</i> (Peredamiranda and Delgado, 1990; Almtorp <i>et al.</i> , 1991) <i>H. crenata</i> (found in the present work but with different molecular weight).	Antinociceptive and Anti-inflammatory (Magalhaes <i>et al.</i>)
Arucadiol / hydroxymiltirone (or isomer)	C ₁₉ H ₂₂ O ₃		298.1459	<i>H. crenata</i> (present work)	Not mentioned
Benzyl disaccharide	C ₁₈ H ₂₆ O ₁₀		402.187	<i>H. crenata</i> (present work)	Not mentioned
Benzyl pentoside derivative	C ₁₂ H ₁₆ O ₅	Not found	240.0891	<i>H. crenata</i> (present work)	Not mentioned
Borneol <i>endo-1,7,7-Trimethyl-bicyclo[2.2.1]heptan-2-ol</i>	C ₁₀ H ₁₈ O		154.2	<i>H. emoryi</i> <i>H. crenata</i> (Sheth <i>et al.</i> , 1972; Pino <i>et al.</i> , 2002) and present work.	Convulsant* (Quintans <i>et al.</i>) Anti-inflammatory (Juhas <i>et al.</i> , 2008) Antinociceptive (Martinez <i>et al.</i> , 2009) Modulator of GABA action* (Granger <i>et al.</i> , 2005) HA and 5-HT in rat's hypothalamus (Li <i>et al.</i> , 2004) Inhibitors on N-(3,5-dichlorophenyl) succinimide nephrotoxicity* (Hong <i>et al.</i> , 1999)

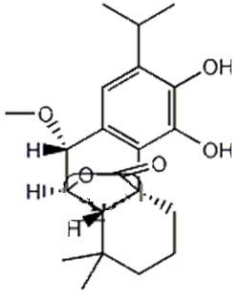
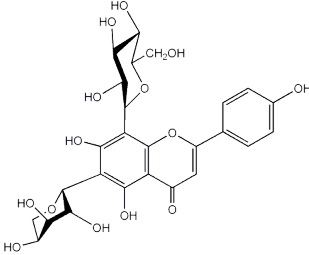
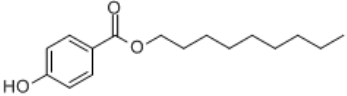
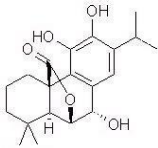
Compound	Molecular formula	Structure	Molecular weight/ (g/mol)	<i>Hyptis</i> Species/Author	Pharmacological effects Applied for the compound published.
Camphor <i>1,7,7-Trimethylbicyclo[2.2.1]heptan-2-one</i>	C ₁₀ H ₁₆ O		152.2	<i>H. suaveolens</i> <i>H. emoryi</i> <i>H. crenata</i> (Sheth <i>et al.</i> , 1972; Scramin <i>et al.</i> , 2000; Joy <i>et al.</i> , 2008; Rebelo <i>et al.</i> , 2009) and present work.	Hypotension and myocarditis (Hempel <i>et al.</i> , 2005; Bhaya and Beniwal, 2007b) Antinociceptive (Taniguchi <i>et al.</i> , 1994) Anti-inflammatory (Yoon <i>et al.</i>) Antiproliferative and antitumor (Loizzo <i>et al.</i>) Antispasmodic activity (Astudillo <i>et al.</i> , 2004) Enhancement of neutrophil activity (Chao <i>et al.</i> , 2005)
Carnosic acid	C ₂₀ H ₂₈ O ₄		332.4	<i>H. dilatata</i> (Urones <i>et al.</i> , 1998) <i>H. crenata</i> (present work)	Antinociceptive and Anti-inflammatory (Qnais <i>et al.</i>) NF Kappa B Activities (anti-inflammatory)* (Yu <i>et al.</i> , 2009) Cytotoxic activities (Costa <i>et al.</i> , 2007; Nga <i>et al.</i> , 2007) Activators of the human peroxisome proliferator-activated receptor gamma (Rau <i>et al.</i> , 2006)
Carnosol <i>1,3,4,9,10,10aS-hexahydro-5,6-dihydroxy-1,1-dimethyl-7-isopropyl-2H-9S,4aR-(epoxymethano)phenanthren-12-one</i>	C ₂₀ H ₂₆ O ₄		330.4	<i>H. dilatata</i> <i>H. martiusii</i> (Urones <i>et al.</i> , 1998; Costa-Lotufo <i>et al.</i> , 2004) <i>H. crenata</i> (present work)	Anti-inflammatory (Poeckel <i>et al.</i> , 2008) NF-Kappa B inhibition* (Anti-inflammatory) (Lian <i>et al.</i>) Neuroprotective effects (Tamaki <i>et al.</i> ; Izumi <i>et al.</i> , 2007)* (Glutathione metabolism) Inhibitor of transporter P-glycoprotein* (Nabekura <i>et al.</i>) Prostate cancer* (Johnson <i>et al.</i> , 2008) Gastroprotective (Areche <i>et al.</i> , 2009a) Activation of gamma receptor (implying an anti-inflammatory potential) (Poeckel <i>et al.</i> , 2008)*

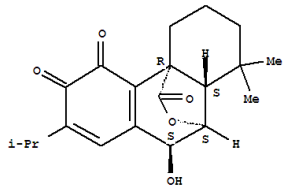
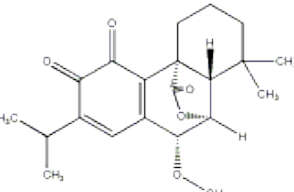
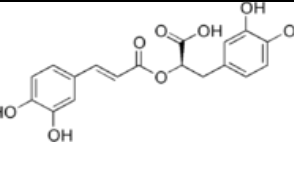
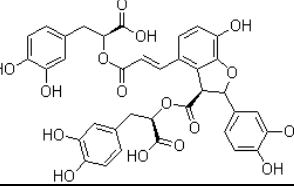
Compound	Molecular formula	Structure	Molecular weight/ (g/mol)	<i>Hyptis</i> Species/Author	Pharmacological effects Applied for the compound published.
Clemomandshuricoside B (or isomer)	C ₂₂ H ₃₀ O ₁₃	Not found	502.2	<i>H. crenata</i> (present work)	Not mentioned
Coumaroyl hexoside	C ₁₅ H ₁₈ O ₈	Not found	326.1	<i>H. crenata</i> (present work)	Anti-inflammatory and antinociceptive activities (Kupeli <i>et al.</i> , 2007)
Creoside I (or isomer)	C ₁₄ H ₂₄ O ₇	Not found	304.2	<i>H. crenata</i> (present work)	Not mentioned
5-Deacetoxy-5'-epiolguine (or isomer)	C ₁₆ H ₂₀ O ₇	Not found	324.11	<i>H. crenata</i> (present work)	Not mentioned
Diterpenoid derivative	C ₁₉ H ₂₂ O ₅	Not described ^A	330.1546	<i>H. crenata</i> (present work)	Not described ^A
Diterpenoid derivative	C ₂₀ H ₃₀ O ₄	Not described ^A	334.2216	<i>H. crenata</i> (present work)	Not described ^A
Diterpenoid	C ₂₀ H ₂₈ O ₃	Not described ^A	316.2112	<i>H. crenata</i> (present work)	Not described ^A
Diterpenoid	C ₂₀ H ₃₂ O ₆	Not described ^A	368.2	<i>H. crenata</i> (present work)	Not described ^A
Diterpenoid	C ₂₀ H ₃₀ O ₄	Not described ^A	334.2	<i>H. crenata</i> (present work)	Not described ^A
Diterpenoid	C ₂₀ H ₂₄ O ₇	Not described ^A	376.1414	<i>H. crenata</i> (present work)	Not described ^A
Diterpenoid	C ₂₀ H ₃₂ O ₄	Not described ^A	336.2191	<i>H. crenata</i> (present work)	Not described ^A
Epoxyabietrienone methyl ether derivative	C ₂₁ H ₂₈ O ₆	Not found	376.1774	<i>H. crenata</i> (present work)	Not described ^A
Epoxykaurene derivative	C ₂₂ H ₃₀ O ₇	Not found	406.188	<i>H. crenata</i> (present work)	Not mentioned
Epoxyignan derivative	C ₁₈ H ₂₀ O ₇	Not found	348.126	<i>H. crenata</i> (present work)	Cytotoxic and Antioxidative activities(Duan <i>et al.</i> , 2009)
Esquirolin B / suaveololi (or isomer)	C ₂₀ H ₃₄ O ₂	Not found	306.2633	<i>H. dilatata</i> (Urones <i>et al.</i> , 1998) <i>H. crenata</i> (present work)	Not mentioned
Ethylrosmanol / <i>epi</i> -ethylrosmanol (or isomer)	C ₂₂ H ₃₀ O ₅	Not found	374.1983	<i>H. crenata</i> (present work)	Anti- HIV activities(Paris <i>et al.</i> , 1993)

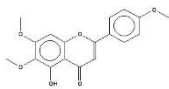
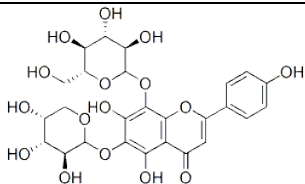
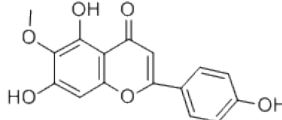
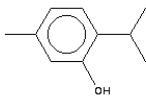
Compound	Molecular formula	Structure	Molecular weight/ (g/mol)	<i>Hyptis</i> Species/Author	Pharmacological effects Applied for the compound published.
Eucalyptol (1,8 cineole)	C ₁₀ H ₁₈ O		154.2	<i>H. recurvata</i> <i>H. emoryi</i> <i>H. fruticosa</i> <i>H. crenata</i> <i>H. martiusii</i> <i>H. mutabilis</i> <i>H. spicigera</i> <i>H. suaveolens</i> (Fragoso-Serrano <i>et al.</i> , 1999; Leclercq <i>et al.</i> , 2000; Scramin <i>et al.</i> , 2000; Azevedo <i>et al.</i> , 2001; Azevedo <i>et al.</i> , 2002; Campos <i>et al.</i> , 2002; Aguiar <i>et al.</i> , 2003; Araujo <i>et al.</i> , 2006; Koba <i>et al.</i> , 2007; Menezes <i>et al.</i> , 2007; Ngassoum <i>et al.</i> , 2007; Noudjou <i>et al.</i> , 2007b; Botrel <i>et al.</i> , 2009; Rebelo <i>et al.</i> , 2009) <i>H. crenata</i> (present work)	Antinociceptive* (Bastos <i>et al.</i> , 2009; Martinez <i>et al.</i> , 2009) Anti-Inflammatory* (Bastos <i>et al.</i> ; Juergens <i>et al.</i> , 2003) Sedating and antipyretic activity (Ortiz de Urbina <i>et al.</i> , 1989)
Eudesmenone or labdanolide derivative	C ₁₇ H ₂₆ O ₄	Not found	294.1722	<i>H. crenata</i> (present work)	Not mentioned
Germacradiene derivative (salviadienol B or isomer)	C ₂₈ H ₄₂ O ₁₁	Not found	554.2621	<i>H. crenata</i> (present work)	Not mentioned
Hydroxyabdanolidoic acid derivative (norambreinolide-18,6-olide or isomer)	C ₁₆ H ₂₂ O ₄	Not found	278.1589	<i>H. crenata</i> (present work)	Not mentioned
Hydroxyluteolin pentoside	C ₂₀ H ₁₈ O ₁₁		434.092	<i>H. crenata</i> (present work)	Not mentioned

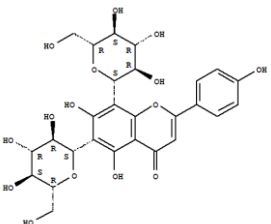
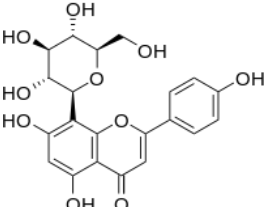
Compound	Molecular formula	Structure	Molecular weight/ (g/mol)	<i>Hyptis</i> Species/Author	Pharmacological effects Applied for the compound published.
Hyptol	C ₂₀ H ₂₆ O ₄			<i>H. fruticosa</i> (Monache et al., 1977) <i>H. crenata</i> (present work)	Not mentioned
Isoschaftoside	C ₂₆ H ₂₈ O ₁₄		564.1551	<i>H. crenata</i> (present work)	Not mentioned
Isovitexin	C ₂₁ H ₂₀ O ₁₀		432.1132	<i>H. crenata</i> (present work)	Antinociceptive and anti-inflammatory(Rabelo <i>et al.</i> , 2013)
Labdanone / kaurenone diterpenoid	C ₂₀ H ₃₂ O ₆	Not found	368.2273	<i>H. crenata</i> (present work)	Not mentioned
Labdenone diterpenoid	C ₂₀ H ₃₂ O ₅	Not found	352.2324	<i>H. crenata</i> (present work)	Not mentioned

Compound	Molecular formula	Structure	Molecular weight/ (g/mol)	<i>Hyptis</i> Species/Author	Pharmacological effects Applied for the compound published.
Luteolin glycoside derivative	C ₂₇ H ₃₀ O ₁₆	 The structure shows a central flavone core (luteolin) with two glucose units attached via glycosidic bonds. The glucose units are in their cyclic pyranose form, with multiple hydroxyl groups indicated by dashed and wedged bonds.	610.2	<i>H. crenata</i> (present work)	Neuroprotective, antinociceptive and anti-inflammatory (Guo <i>et al.</i> , 2013; Rabelo <i>et al.</i> , 2013)
Maoecrystal V (or isomer)	C ₁₉ H ₂₂ O ₅	 The structure is a complex polycyclic terpenoid with multiple oxygen-containing functional groups, including a lactone ring and several hydroxyl groups.	330.1358	<i>H. crenata</i> (present work)	Not mentioned
Martiusane (or isomer)	C ₂₀ H ₃₀ O ₃	Not found	318.2269	<i>H. crenata</i> (present work) <i>H. martiusii</i> (Cavalcante Da Cruz Aragao <i>et al.</i> , 2004)	Not mentioned
Megastigmene glycoside derivative	C ₁₉ H ₃₀ O ₈	Not found	386.2016	<i>H. crenata</i> (present work)	Not mentioned
Megastigmenone glycoside derivative	C ₁₉ H ₃₂ O ₇	Not found	372.2	<i>H. crenata</i> (present work)	Not mentioned
Megastigmadienone glycoside derivative	C ₁₉ H ₃₀ O ₉	Not found	402.1786	<i>H. crenata</i> (present work)	Not mentioned
Methoxynepetaefolin derivative (or isomer)	C ₂₃ H ₃₂ O ₈	Not found	436.199	<i>H. crenata</i> (present work) <i>H. fasciculata</i> (Ohsaki <i>et al.</i> , 2005)	Not mentioned

Compound	Molecular formula	Structure	Molecular weight/ (g/mol)	<i>Hyptis</i> Species/Author	Pharmacological effects Applied for the compound published.
Methyl epirosmanol / oxoinuroyleanol (or isomer)	C ₂₁ H ₂₈ O ₅		360.1834	<i>H. crenata</i> (present work)	Not mentioned
Methyl pisiferate (or isomer)	C ₂₁ H ₃₀ O ₃	Not found	330.2085	<i>H. crenata</i> (present work)	Not mentioned
Neoisoschaftoside	C ₂₆ H ₂₈ O ₁₄		564.2	<i>H. crenata</i> (present work)	Not mentioned
Nonyl hydroxybenzoate derivative	C ₁₆ H ₂₄ O ₃		264.2	<i>H. crenata</i> (present work)	Not mentioned
Pervoside B (or isomer)	C ₁₆ H ₂₀ O ₉	Not found	356.0997	<i>H. crenata</i> (present work)	Not mentioned
Rosmanol	C ₂₀ H ₂₆ O ₅		346.4	<i>H. dilatata</i> (Urones <i>et al.</i> , 1998) <i>H. crenata</i> (present work)	Inhibits lipopolysaccharide-induced inos and COX-2 expression through downregulating MAPK, NF-Kappab, STAT3 and C/EBP signaling pathways* (Anti-inflammatory) (Lai <i>et al.</i> , 2009) Neuroprotective effects (Park, 2009)

Compound	Molecular formula	Structure	Molecular weight/ (g/mol)	<i>Hyptis</i> Species/Author	Pharmacological effects Applied for the compound published.
Rosmaquinone (or isomer)	C ₂₀ H ₂₄ O ₅		344.2	<i>H. crenata</i> (present work)	Not mentioned
Rosmaquinone A or B (or isomer)	C ₂₁ H ₂₆ O ₅		358.2	<i>H. crenata</i> (present work)	Not mentioned
Rosmarinic acid 2 <i>R</i>)-2-[[[(2 <i>E</i>)-3-(3,4-dihydroxyphenyl)-1-oxo-2-propenyl]]oxy]-3-(3,4-dihydroxyphenyl) propanoic acid	C ₁₈ H ₁₆ O ₈		360.3	<i>H. capitata</i> <i>H. verticillata</i> (Heinrich <i>et al.</i> , 1992; Kuhnt <i>et al.</i> , 1995b; Heinrich, 2003) <i>H. crenata</i> (present work)	Antinociceptive (Park, 2009) Anti-inflammatory* (Park, 2009; Rocha <i>et al.</i> , 2009; Sahu and August, 2009) Anticancer (Feng <i>et al.</i> ; Nabekura <i>et al.</i> ; Nabekura <i>et al.</i> ; Stanojkovic <i>et al.</i> ; Yesil-Celiktas <i>et al.</i> ; Scheckel <i>et al.</i> , 2008; Koeberle <i>et al.</i> , 2009; Kwak <i>et al.</i> , 2009; Xavier <i>et al.</i> , 2009) Suppresses retinal neovascularisation (Kim <i>et al.</i> , 2009)
Salvianolic acid B, E or L (or isomer)	C ₃₆ H ₃₀ O ₁₆		718.2	<i>H. crenata</i> (present work)	Neuroprotective, anti-inflammatory and antidepressant (Chen <i>et al.</i> , 2011; Ye <i>et al.</i> , 2011; Feng <i>et al.</i> , 2012)
Salvidorol (or isomer)	C ₁₉ H ₂₆ O ₄	Not found	318.2	<i>H. crenata</i> (present work)	Not mentioned

Compound	Molecular formula	Structure	Molecular weight/ (g/mol)	<i>Hyptis</i> Species/Author	Pharmacological effects Applied for the compound published.
Salvigenin (4 <i>H</i> -1-benzopyran-4-one, 5-hydroxy-6,7-dimethoxy- 2-(4-methoxyphenyl)-	C ₁₈ H ₁₆ O ₆		328.3	<i>H. urticoides</i> (De Vivar <i>et al.</i> , 1991) <i>H. crenata</i> (present work)	Anticholinesterase (Kolak <i>et al.</i> , 2009) Anticancer (Kamatou <i>et al.</i> , 2008)
Salviregnone (or isomer)	C ₁₉ H ₂₄ O ₂	Not found	284.2	<i>H. crenata</i> (present work)	Not mentioned
Schaftoside	C ₂₆ H ₂₈ O ₁₄		564.2	<i>H. crenata</i> (present work)	Antihepatotoxic effects(Hoffmannbohm <i>et al.</i> , 1992)
Scutellarein <i>O</i> -methyl ether	C ₁₆ H ₁₂ O ₆		300.1	<i>H. crenata</i> (present work)	Anti-inflammatory(Rao <i>et al.</i> , 2009)
Scutellarein di- <i>O</i> -methyl ether	C ₁₇ H ₁₄ O ₆	Not found	314.1	<i>H. crenata</i> (present work)	Anti-inflammatory(Williams <i>et al.</i> , 1999)
Sesquiterpenoid derivative	C ₁₅ H ₂₆ O ₃	Not found	254.1779	<i>H. crenata</i> (present work)	Not mentioned ^A
Sesquiterpenoid derivative	C ₁₅ H ₂₄ O ₃	Not found	252.1802	<i>H. crenata</i> (present work)	Not mentioned ^A
Thymol (Phenol, 5-methyl-2-(1-methylethyl)-	C ₁₀ H ₁₄ O		150.2	<i>H. mutabilis</i> <i>H. verticillata</i> (Kuhnt <i>et al.</i> , 1995a; Aguiar <i>et al.</i> , 2003; Heinrich, 2003) <i>H. crenata</i> (present work)	Antinociceptive (Angeles-Lopez <i>et al.</i> ; Angeles-Lopez <i>et al.</i> , 2010) Anti-inflammatory (Braga <i>et al.</i> , 2006; Monteiro <i>et al.</i> , 2007) Gastroprotective (Monteiro <i>et al.</i> , 2007) Cytotoxic (Ersev <i>et al.</i> , 1999) Acetylcholinesterase* (Andres and Narayanaswamy, 1997; Jukic <i>et al.</i> , 2007)
Trilobiol (or isomer)	C ₂₁ H ₂₆ O ₇	Not found	780.3	<i>H. crenata</i> (present work)	Not mentioned
Triterpenoid	C ₃₀ H ₄₆ O ₇	Not found	518.3	<i>H. crenata</i> (present work)	Not described ^A

Compound	Molecular formula	Structure	Molecular weight/ (g/mol)	<i>Hyptis</i> Species/Author	Pharmacological effects Applied for the compound published.
Unassigned compound	C ₁₂ H ₂₀ O ₄	Not described ^A	228.1	<i>H. crenata</i> (present work)	Not mentioned ^A
Unassigned compound	C ₂₁ H ₃₂ O ₈	Not described ^A	412.2	<i>H. crenata</i> (present work)	Not described ^A
Unassigned compound	C ₁₂ H ₂₀ O ₄	Not described ^A	228.1258	<i>H. crenata</i> (present work)	Not described ^A
Unassigned compound	C ₁₂ H ₂₀ O ₄	Not described ^A	228.1258	<i>H. crenata</i> (present work)	Not described ^A
Unassigned compound	C ₁₉ H ₁₆ O ₃	Not described ^A	292.1176	<i>H. crenata</i> (present work)	Not described ^A
Vicenin-2 (or isomer)	C ₂₇ H ₃₀ O ₁₅		594.1652	<i>H. crenata</i> (present work)	Antinociceptive and anti-inflammatory (Gorzalczany <i>et al.</i> , 2011 {Marrassini, 2011 #1779})
Vitexin	C ₂₁ H ₂₀ O ₁₀		432.1131	<i>H. crenata</i> (present work)	Antinociceptive and anti-inflammatory (Rabelo <i>et al.</i> , 2013)

Not described^A = Not possible to describe due to unspecific identification of the compounds

Table appendix A – Compounds identified in the *Hyptis crenata* decoction extract and fractions that were tested in this PhD project

Appendix B – Traditional Use Questionnaire

Questionnaire applied in the traditional use survey

I – When did you first use *H.crenata*?

II – How often do you use it?

III – Do you think of *H.crenata* as a regular drink or tonic (like coffee)?

IV – What are the situations when you use or are using *H.crenata*?

V – What effects do you think that *H.crenata* has?

VI – How do you feel after you have consumed it?

VII – Are you using *H.crenata* to treat an illness, if so which and is it proving an effective treatment?

VIII – Did you take or are you taking any other medication when you were or are using *H.crenata*? If yes, which kind? E.g. herbal

IX – How much are you taking? How many times are you taking *H.crenata* per day? What time of the day do you use *H.crenata*?

X – How frequently and over what time scale have you been using it? Days, weeks, months or years?

XI – Describe any symptoms or side effects you may experience through use of *H.crenata*?

XII – Description of preparation process and utilisation:

XIII – How do you use *H.crenata*? e.g.: decoction (tea), tinctures, syrup, paste, infusion, ointment, salad (e.g. eat the leaves).

XIV – Which part of the plant is used? e. g. stems, leaves, flowers, whole plant, roots.

XV – How much material is used during preparation? E.g. numbers or grams of leaves, stems or flowers.

XVI – How do you use the material collected? E.g. fresh, dried

XVII – Do you store the material for use? If yes, how do you store it? How long do you store it for?

XVIII – Is there any particular time of the day that you collect the plant? E.g. morning, afternoon or evening.

XIX – Is there any particular time of the year that you collect the plant? E.g. January...December

XX – Where did you get the *H.crenata* from?

XXI – From whom did you learn how to prepare *H.crenata*?

XXII – Which type of solvent is used? e.g water, alcohol, honey... sugar.

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