Chapter

Methods for Evaluating the *In vivo* Analgesic and Anti-inflammatory Activity of Essential Oils

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Abstract

Essential oils (EOs) are products of the secondary metabolism of plants, and the constituents are mainly monoterpenes and sesquiterpenes of formula general $(C_5H_8)n$. The beneficial effects of the volatile compounds of essential oils have been used for a very long time by ancient civilizations to treat common pathologies. Today, so-called natural medicines are enjoying growing success with the public. Numerous studies have demonstrated that the essential oil has significant potential as antibacterial, antifungal, antioxidant, antidiabetic and painkiller agents. The use of animals is widespread in biomedical research and pain studies are no exception. Since experimentation on human subjects must be limited to moderate stimuli that do not cause injury or disease, the researchers turned to animals to find answers to their questions. Several methods have been used for the evaluation of the anti-inflammatory activity of medicinal plant extracts, and most of the tests used to evaluate pain and inflammation in animal experiments involve inducing pain in animals with different agents.

Keywords: essential oil, medicinal plants, animal experimentation, nociception, rodents, analgesic activity, anti-inflammatory activity

1. Introduction

Definitely, pain has been defined by the International Association for the Study of Pain (IASP, 1979) as an 'unpleasant sensory and emotional experience related to actual, potential, or described tissue damage by the patient ' [1, 2]. Typically, it is triggered by noxious stimuli and transmitted *via* specialised neural networks to the central nervous system (CNS), where it is interpreted as such. It is also a way of protecting the body [3]. In this regard, pain and inflammation remain the most important and devastating health problems, affecting 80% of the world's population [4]. They are considered a major environmental health problem, affecting all countries. [5]. Further, untreated, persistent and prolonged pain is the most common problem, causing both physical damage and psychological distress [6]. Likewise, pain can be caused by a variety of factors, inclusive of injury, illness and psychological factors [3].

More to the point, the mechanisms of pain and inflammation, together with the modes of action of treatments, have shown to be numerous and complex. Some are still little known. Thus, treatments, classified into three levels (non-opioid, weak opioid and strong opioid), are often combined and provide relief for a large proportion of patients [7]. In this regard, analgesics or painkillers are designed to reduce or abolish painful sensations without causing loss of consciousness or suppressing other sensitivities [8]. Likewise, anti-inflammatory medicines are symptomatic drugs that do not act on the cause. They are indicated when inflammation, a normal process of defence against aggression, becomes bothersome, particularly because of pain [9]. In virtue of which, they are very widely used in a large number of diseases and more specifically in the presence of inflammation, the same as in rheumatology [3].

More and more, alternative therapies are unconventional methods of pain relief that do not involve drugs or surgery. Alternative therapies include acupuncture, massage therapy and chiropractic care. Hence, these therapies are often used in conjunction with traditional medical treatments [3]. Traditional medicine has strong cultural roots, with many plants used to treat pain. This ancestral knowledge can be regarded as a source of inspiration for finding numerous active ingredients and as a consequence enabling therapeutic innovation in the management of pain and inflammation [10].

2. Essential oils

The active molecules, involved in plant defence mechanisms, are derived from secondary metabolism. Besides, they are not directly involved in plant growth but have evolved to provide natural protection against attacks by microbes or insects [11]. Above and beyond, some of such secondary metabolites are concentrated in the oil sacs, which are essential oil-secreting pockets [12]. In consequence, exploring essential oils for molecules with biological activity seems to be an interesting avenue.

2.1 Definition

The eighth edition of the French Pharmacopoeia defines EOs as 'products of generally fairly complex composition containing volatile products contained in plants and more or less modified during preparation'. There are various processes for extracting these volatile principles. At the time, EOs were also referred to as 'essences' or 'volatile oils'. Since the ninth edition (1972), the Pharmacopoeia now only uses the term 'essential oil' [13–15]. Additionally, essential oils have shown to be complex natural mixtures of volatile secondary metabolites, isolated from plants by hydrodistillation or mechanical expression [16]; they are obtained from leaves, seeds, buds, flowers, twigs, herbs, bark, wood, roots or fruit, but alike from the gums that run off the trunks of trees. Above and beyond, hydrodistillation is still the most widely used method of producing essential oils, in particular for commercial purposes [17]. In addition, secondary metabolites are extracted from plants by steam distillation. The volume of essential oil recovered depends on the distillation yield, which varies for the same plant depending on the season [18]. Likewise, essential oils can be obtained by cold expression, as in the case of citrus fruits. New techniques have been developed to increase production yields, in respect such as extraction using liquid carbon dioxide at low temperature and high pressure [19] or extraction assisted by ultrasound or microwaves [20].

2.2 Chemical composition of essential oils

Contrary to what might be suggested by its name, a pure and natural essential oil does not contain any fats. It is made up of molecules with a carbon skeleton. As well, essential oils represent complex mixtures that may contain more than 300 different compounds [21–24]. In virtue of which, such compounds are volatile molecules, the vast majority of which belong to the terpene family. Only the most volatile terpenes, that is. those whose molecular weight is not too high, are used [23]. Terpene compounds are products of secondary metabolism. There are mainly monoterpenes (C_{10}) and sesquiterpenes (C_{15}). Several thousand compounds have been described and have shown to be classified according to their number of rings (acyclic, mono- and bicyclic compounds) together with the nature of the functions they carry (alcohols, aldehydes, ketones, ether-oxides) [25].

2.3 Physico-chemical properties of essential oils

In general, essential Oils are colourless or pale yellow in their liquid state at ordinary temperatures. However, all EOs are volatile, fragrant and flammable; their specific gravity is usually less than one [26]. Only three officinal EOs have a specific gravity greater than that of water: cinnamon, clove and sassafras. They are sparingly soluble in water, soluble in alcohols and in most organic solvents. They are alterable and are very sensitive to oxidation [27].

Essential oils share organoleptic properties (characteristics of a substance that can be perceived by the sense organs: flavour, odour, appearance and consistency of the object) such as being liquid at room temperature, volatile and water vapour permeable [28].

Modern scientific work has made it possible to better understand the essences and to precisely define their different constituents and their physico-chemical characteristics, revealing the principle of their long-known therapeutic action. The physiological role of essential oils in the herb kingdom remains unknown. However, the molecular diversity of the metabolites they contain endows them with a wide range of biological roles. Additionally, several essential oils, such as cinnamon, cayenne pepper, bay leaf and oregano oils, have antioxidant properties [29, 30].

An anti-inflammatory effect has been described for essential oils of citrus cultivars. The results suggest that *C. japonica* and *C. maxima* are promising candidates for relieving inflammatory diseases. These research findings provide the scientific basis for using essential oils from citrus cultivars to reduce inflammatory symptoms[31]. Above and beyond, the antifungal activities of numerous essential oils, including thyme, citronella, cinnamon and tea tree oils [17], have been described. The efficacy of oils extracted from yarrow, *Achillea fragrantissima* [32] and *A. milefolium* [33] against the pathogenic yeast *Candida albicans*, has also been demonstrated.

Certain essential oils have anti-tumour activity and are used in the preventive treatment of certain types of cancer. The essential oil, isolated from the seeds of *Nigella sativa* L., demonstrates cytotoxic activity *in vitro* against various tumour cell lines. *In vivo*, it limits the proliferation of liver metastases and delays the death of mice that have developed the p815 tumour [34].

2.4 Analgesic and anti-inflammatory activity of essential oils

A number of pain-relieving essential oils can help us to overcome certain ailments, in respect such as dental pain, headaches or chronic inflammation. The essential oils

presented below can effectively be used to fight pain. Most of them have analgesic, analgesic and anaesthetic mechanisms of action. Lemon eucalyptus essential oil acts as an anti-inflammatory thanks to its citronellal content of over 65%, which helps to relieve pain. Clove essential oil is one of the ultimate pain-relieving essential oils. Besides, its eugenol content of over 80% gives it formidable analgesic properties. It is recommended for relieving dental pain, and peppermint essential oil has multiple properties that provide fast and effective pain relief. Firstly, it acts as a local analgesic not only thanks to its menthol content but also as a mild anaesthetic [35].

The bioactive constituents of essential oil extracted from many medicinal plants were known to provide protection against prolonged inflammation and improve the health of mortals. In this regard, the anti-inflammatory properties of these plants are of immense importance to the drug discovery process [36, 37]. Specifically, lavender essential oil has been shown to inhibit inflammation by inhibiting the nascence of TNF (tumour necrosis factor) and NF-kB (nuclear factor kappa–light chain enhancer of activated B cells) in the murine brain and human umbilical vein endothelial cells. [38]. Linalool and cinnamaldehyde present in native cinnamon leaf essential oil were found to be anti-inflammatory against endotoxin introduced into mice [39].

3. Experimental animals

Healthy adult Swiss albino mice of both sexes (20 to 35 g and 6 to 8 weeks old) are used for testing. All mice are fed commercial pellets and must have access to water ad libitum, alternately. Mice must be acclimated one week before experience in all procedures to minimise stress. All mice used in studies should be handled according to internationally accepted standard guidelines for the use of laboratory animals [39, 40].

4. Extraction of essential oils

Essential oils can be extracted by methods of steam distillation, steam and water utilised strategies to isolate these aromatic essences[41] The best extraction method will undoubtedly be one which does not use any solvent, generates no residual waste and requires no energy to process it. Obviously, there is no other solvent more ideal than water on plants. It is available, recyclable and non-toxic. Water is already widely used in the extraction of essential oils by steam distillation[42].

5. Acute toxicity test

Characteristically, in the presence of an unknown substance, the first step in the search for pharmacological activity begins with a toxicological study and in particular, the evaluation of the lethal dose 50 (LD 50), that is. the dose, which causes the mortality of 50% of the animals. In virtue of which, increasing doses of extracts are administered to rats and mice until mortality is achieved. Although this technique is highly controversial from an ethical point of view, it nevertheless provides high-quality information:

1. Firstly, it determines the toxicity of the substance and the therapeutic margin; in other words, the ratio between the active dose and the toxic dose for the animal

species tested; this is an essential step in the use of any substance for therapeutic purposes.

2. Observation of the first symptoms of toxicity reveals the target organs; in other words, those which are preferentially affected by the toxicant; toxicity stands alike for an excellent criterion for orienting research into pharmacological activity [41].

More to the point, essential oils are not products that can be used without risk. Similarly as all natural products, 'just because it is natural does not mean it is safe for the body' [23]. The acute toxicity test for essential oil extracts can quickly be carried out using the method described by the organisation for economic cooperation and development (OECD) [43]. This test consists of administering experimental doses to the animals and monitor them for signs of poisoning, including drooling, convulsions, unusual activity, loss of consciousness, coma or even death. These observations are performed regularly for up to 48 hours [44].

6. Methods used for assessment of the analgesic and anti-inflammatory activity *in vivo*

The use of animals is widespread in biomedical research, and pain studies are no exception. As experiments on human subjects have to be limited to moderate stimuli that do not cause injury or disease, researchers have turned to animals to find answers to their questions [45].

Nociception and pain constitute a vast area of neuroscience and medical scientific research. As we know, the use of animals in scientific research has been controversial since ancient times [46], although these animal models have great merit in the advancement of biomedical sciences through their important contributions to our growing understanding pathological and biological processes [47]. Over time, various tests and models have been developed in rodents to provide fundamental and translational research tools on this subject; to study pain and try to reduce it, tests using thermal, mechanical and chemical stimuli, Hyperalgesia and pain measurements, and inflammatory or neuropathic pain models constitute one of the most important tools available to researchers in this area [48]. Preclinical therapeutic research should consequently combine pain models with nociceptive tests in order to be more relevant [49].

6.1 Methods used to study the analgesic activity *in vivo*

Most of the tests used to assess pain in animal experimentation involve inducing pain in animals using different agents [50]. Further, the tests are grouped around three basic types of pain: thermal nociception, chemical nociception and mechanical nociception. Nevertheless, in the following figure, we describe and critically analyse the most commonly used behavioural tests of nociception in animals (**Figure 1**) [49].

6.1.1 Thermal nociception

In mammalian nociceptors, noxious heat above 40°C activates thermosensitive C fibres and heat above 52°C activates A fibres [51]. In addition, tests measuring

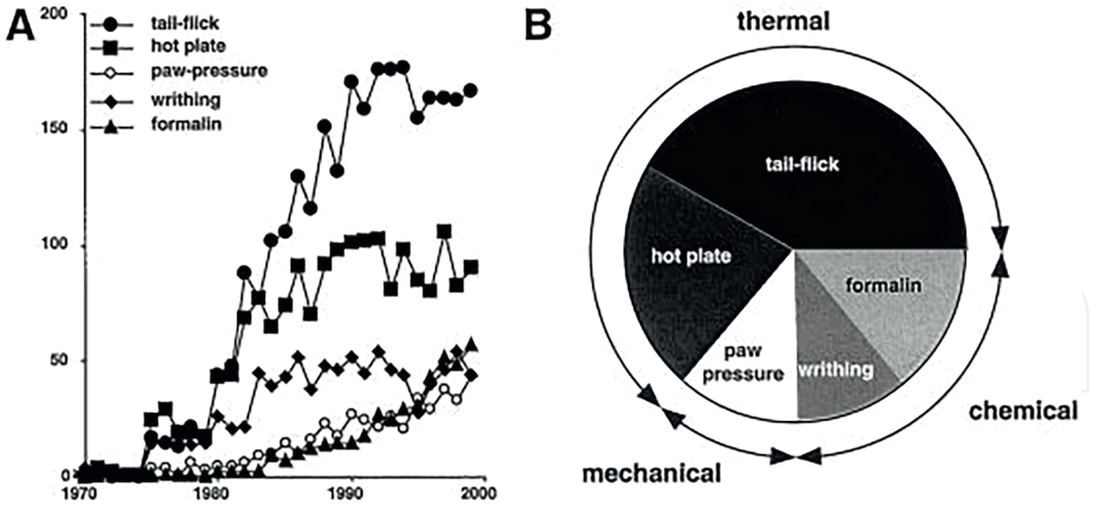

Figure 1.
A. A number of original articles published between 1970 and 1999 in which researchers used one of the five most common pain sensation tests, B. The relative proportions of these categories of articles appearing during the year 1999 (based on Medline) [49].

the nociceptive response to heat can be used experimentally in both rats and mice. Likewise, the stimulus can stop automatically when the animal responds [48, 49]. The animal is placed inside the heating plate and waited a few moments for it to react to the pain. The mouse must be recovered immediately after observing its response to the experiment to avoid any risk of burns. We can do the same experiment and repeat it several times to check the values, but this is often observed in some laboratory animals, either stress or habit of the protocol gives us different values. Repeating measurements for the same mouse may lead to different results. [49, 50]. There are also some limitations to this test, repeated measurements lead to learning phenomena, and these lead to variations in reaction latency [52].

When we touch something too hot or too cold, our senses translate this into a sensation of pain. If you put your hand in a fire, the resulting burning sensation will cause your body to move your hand away as quickly as possible. Feeling pain is actually proof that your body is working hard to keep you safe. So, losing the ability to do that means you find yourself in real trouble.

The hot plate and tail-flick tests measure an animal's ability to consciously remove a part of its body from a heat source, and they all test the ability to an animal to feel and respond to a certain degree of painful stimulation[52].

6.1.1.1 Tail withdrawal test, D'Amour and Smith test or Tail-flick *test*

It stands for a simple method that measures a spinal nociceptive reflex. The tail-flick test includes two types that are superficially similar but physically very different [53]. The first consists of immersing the animal's tail in water at a certain temperature. The second type involves applying radiant heat to a specific small area of the tail. More to the point, the surfaces stimulated can be very different. In fact of matter, it is surprising that authors generally consider these two tests to be equivalent [49]. The time taken for the animal to withdraw its tail is measured [49, 54]. Besides, to minimise the risk of tissue trauma due to exposure to heat, a time limit such as 10 seconds is set, at which point the animal is removed from the test[52].

6.1.1.2 Hot plate *test*

The hot plate method relies on measuring latency to assess skin sensitivity to pain. The response to pain usually involves licking the area to relieve the pain, shaking or immediately jumping off the hot plate [53]. Definitely, the hot plate is an open cylindrical space, the base of which is a heatable metal plate. The mouse is placed inside the plate that is preheated to a constant temperature. The animal is monitored and the response time to any type of behaviour is measured, namely paw licking and jumping. Both are considered to be integrated supraspinal responses [49, 53].

The aim was to verify the ability of the products tested to protect the animal against thermal pain. The extracts were administered either through oral or peritoneal way. The animal is placed on a heated metal plate maintained at between 52 and 55°C (**Figure 2**). The latency time for the appearance of behavioural responses is measured, with the animal licking itself, shaking its legs and/or jumping [55].

6.1.2 Chemical nociception

Chemical nociception refers to a nociceptive neuron that expresses receptors capable of detecting noxious, irritating or harmful chemicals[56, 57].

Chemociception, the detection of tissue-damaging chemicals, is important in protecting organisms from tissue damage. The ability of sensory neurons to detect potentially harmful chemicals is based on the activation of pain receptors in various animals with irritating chemical compounds. The ability of nociceptors to detect a variety of seemingly unrelated chemicals was mysterious until it was discovered that the nociceptor-specific TRPA1 ion channel (the transient receptor potential cation channel (TRP) family of receptors) could be activated by many of these chemicals [51, 56, 57].

6.1.2.1 Formalin test

Subcutaneous or intra-plantar injection of formalin into the paw of rats or mice. The time the animal spends licking and biting its paw is measured [58].

Figure 2.
The hot plate test: Latency of hind paw pain response is licking and shaking or jumping.

6.1.2.2 Abdominal contraction test, Koster test or Writhing test

The intraperitoneal administration of serosal irritants causes highly stereotyped behaviour in mice and rats [59]. We notice a decrease in the animal's motor activity, a lack of motor coordination, twisting of the dorsal abdominal muscles, abdominal contractions and the entire body (especially the hind legs), (**Figure 3**). The test goes by several names, including the abdominal torsion test, abdominal contraction response or stretch test, but it is more commonly known as the 'torsion test' [49].

In fact, the torsion test is a chemical method used to induce peripheral pain by injecting irritants, such as phenylquinone or acetic acid into mice. Further, the low frequency of contortions makes it possible to deduce the potency and analgesic activity of the active substance. Manifestations of abdominal torsion in mice were first reported by Siegmund et al.[60], as an arching of the back, extension of the hind limbs and contraction of the abdominal muscles [61]. Likewise, the number of abdominal contractions and body stretches are measured [49, 55, 59, 62, 63]. Hence, these methods have the advantage of being able to demonstrate the effects produced by weak analgesics[64].

6.1.3 Mechanical nociception

Provide responses to stimuli of excessive pressure or mechanical deformation, as well as to breaks in the skin surface (cuts, incisions). These nociceptors, which are often polymodal, have shown to be sensitive to both mechanical deformations and thermal stimuli [56].

6.1.3.1 Von Frey filaments

The von Frey test is a test of touch sensitivity using von Frey filaments, which can vary in diameter. It is considered the most important test and is almost the only mechanical test that can be used reliably not only in rats but also in mice. In rodents, they are mostly used on the plantar surface, while the animal is on a grid. The expected response is withdrawal of the paw. Rodents exhibit a withdrawal reflex when their paw is touched unexpectedly, indicating the degree of their sensitivity. Filaments of different calibres are applied [52, 64]. The von Frey test allows the

Figure 3.
Writhing test: The behaviour is described as the paw stretch and contraction of the abdominal muscles.

response of the two hind legs to be differentiated, and the threshold values are stable over time, allowing repeated measurements [50, 64]. The animal's withdrawal threshold is measured in relation to the force exerted by the filament. Mechanical sensitivity test [45, 55, 64]. The von Frey bristles are nylon monofilaments, or von Frey bristles are nylon monofilaments or stiff metal bristles that exert precise levels of force when pressed against the skin. They can be used to measure mechanical stimulation.

6.1.3.2 Paw pression

The Randall and Selitto test [65] is based on determining the pain threshold induced by the application of pressure. Using a mechanical stimulator, constant or, more often, increasing pressure is applied to the animal's hind limb. The animal's behaviour is assessed: it freezes, withdraws its limb and emits cries. Electromyographic recordings of nociceptive reflexes can alike be made [49].

From all these tests, the tail withdrawal and hot plate tests remain the most commonly used. On the other hand, it should be noted that the rate of publications concerning the tail withdrawal, hot plate and torsion tests stabilised in the 1990s. In contrast, the number of articles based on the formalin test and various tests involving paw withdrawal from mechanical stimuli was noted to have increased[49].

6.1.4 Facial coding scales

A facial expression is one or more movements of the muscles or skin of the face. These movements express the emotional state of the individual to an observer. Therefore, the facial expression of pain can be used as an interesting indicator. If we can estimate these expressions quantitatively using facial coding criteria, this will enable the assessment of pain [66], and recent evidence suggests that facial expressions of pain could alike be used in rodents [67, 68]. Similarly, it is difficult to assess the internal emotional states of rodents by analysing their facial expression. Nevertheless, it has already been used, for example, to assess taste/disgust [69]. In the case of pain expression, the 'grimace scale' defined for mice [67] and rats [68] consists of noting orbital constriction. This grimace scale depends on five facial features: orbital narrowing, nose bulge, cheek bulge, ear position and moustache change. These facial action units have values of 0 (no pain), 1 (mild pain or likely pain) and 2 (severe pain or definitely present) (**Figure 4**)[68, 70]. This new approach to pain assessment in rodents could be facilitated by partial automation.

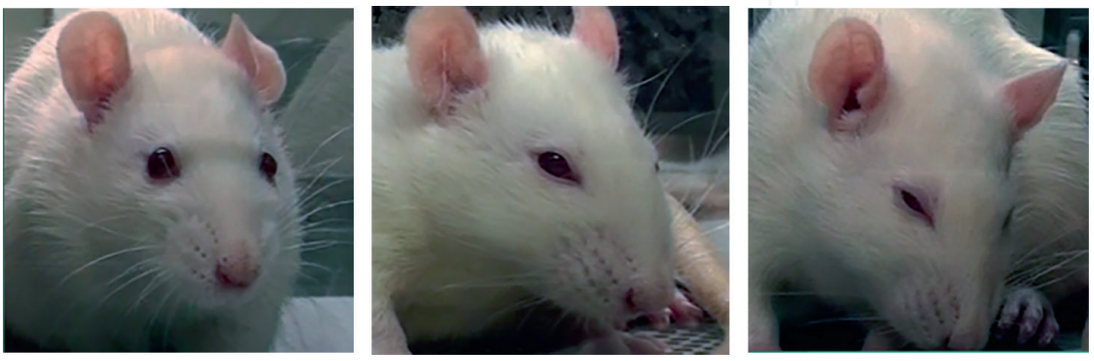

Figure 4.
Examples of visible pain expression on a rat's face, framed from the database and labelled with the rat grimace scale [48, 68] (example adapted from the Facial Pain Expression Table for Rats: Sotocinal et al., 2011).

6.2 Methods used to study the anti-inflammatory activity *in vivo*

The inflammatory response is a physiological process of defence of the body against an attack, which leads to tissue damage. The primary function of the inflammatory response is to eliminate or isolate the attacking agent (bacteria, virus, parasite, damaged tissue) from the rest of the body and to allow, as quickly as possible, tissue repair. The inflammatory response is a physiological process of defence of the body against an attack, which leads to tissue damage. The inflammatory reaction allows the elimination of the aggressors and ensures the repair of the lesions. It stops when the attacks disappear [71] and the inflammatory mediators constitute all the molecules involved in the regulation of the inflammatory process and which activate and sensitize the nociceptive system. In order to relieve inflammatory pain, the body often relies on compounds that stimulate the immune response or on inflammatory mediators themselves [72]. As a consequence, among the tests that are considered a model of short-term inflammatory pain model is the formalin test.

Several methods have been used to assess the anti-inflammatory activity of medicinal plant extracts. In virtue of which, we will illustrate some of the methods used to assess anti-inflammatory activity *in vivo*:

6.2.1 Carrageenan-induced paw oedema

Definitely, Carrageenan-induced paw oedema is certainly one of the most popular tests [72]. Therefore, it is a very sensitive and reproducible test and has been relied upon since ancient times as a model for studying new drugs effective against inflammatory pain [73]. Likewise, carrageenan-induced inflammation enables us to detect orally active acute anti-inflammatory agents. For this reason, it has great predictive value for anti-inflammatory agents that act *via* mediators of acute inflammation[74]. This inflammatory response includes three distinct phases: a first phase involving histamine and 5-hydroxytryptamine, which promote vasodilation, plasma transudation and oedema (0–1 hour), a second phase (1.5–3 hours), which uses kinins as mediators, increases vascular permeability [75]and prostaglandin biosynthesis occurs beyond the third hour (third phase)[76]. A positive effect is explained by the inhibition of the actions or synthesis of pro-inflammatory substances.

Indeed, inflammation occurs when carrageenan is injected. In rodents, intra-plantar injection of carrageenan causes hypersensitivity, which is assessed by mechanical or thermal stimulation. Pain pharmacology: The oedema caused by this photogenic agent can be translated into volume and measured, making it possible to monitor the inflammatory process (**Figure 5**) [77]. Similarly, the behaviours observed can be characterised using a scoring scale [45].

6.2.2 Croton oil-induced ear oedema

The ear oedema is induced by croton oil, using the method of Manga and colleagues. The thickness of the ear is measured using a digital calliper before treatment and a few hours after induction of inflammation [78].

6.2.3 Injection of formalin into the facial region

Injection of formalin into the rat's upper lip: The time the rat spends grooming, scratching and rubbing is assessed [79]. The injection of formalin provokes a biphasic

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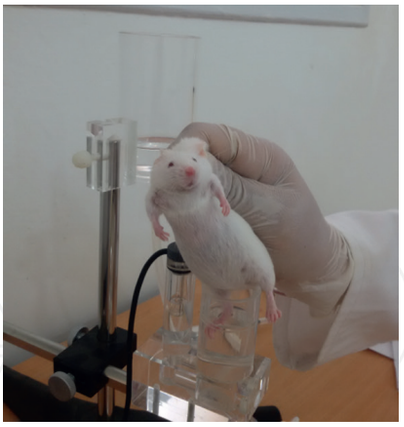

Figure 5.
Measuring the volume of the hind paw of the mice using the plethysmometer.

response similar to the one observed during intra-plantar injection. Administration of analgesics reduces nociceptive behaviour. This test can be used to assess the pain behaviour associated with trigeminal pain, as well as the effects of potentially analgesic drugs [45, 80].

6.2.4 Pulpite

The model stimulating inflammatory pain in the pulpedetar. Intradental injection of capsaicin or formalin in rats. Nociceptive behaviour is measured using a scoring scale. Model simulating inflammatory pain in the dental pulp. Nociceptive behaviour persists for approximately 2 hours. Administration of analgesic reduces the intensity thereof [45].

7. Ethical considerations animal models

If acute pain models or acute pain tests are used in which the pain does not end with the animal's response, the pain should be terminated as quickly as possible. This may mean that the animals must be humanely euthanised as soon as the test is completed (for example, convulsive test) or that analgesics must be administered. Hence, it would be preferable to use avoidance tests rather than tests in which the pain continues after the results have been obtained. Animal testing contributes to life-saving treatments for humans, and in some cases, animals must be used because it is possible that early experiments could cause catastrophic harm if conducted directly on humans. But as cruel and inhumane as animal experiments seem to be, everyone is aware that no animal leaves the laboratory alive, and during most studies, the animals are killed and ultimately dissected. This is why we, as researchers, must use animals in research and teaching, responsibly to protect animals from unnecessary pain and suffering. Researchers should also avoid exposing the animal to stress and fear, which can result from the method of conducting experiments. The number of animals involved should be as minimal as possible, and it is desirable that the duration of the experiment be as short as possible [81].

8. Conclusion

In the light of the facts enlightened above, pain is a complex and subjective phenomenon that can be caused by a variety of factors. Pain relief is an essential aspect of healthcare and is necessary to alleviate suffering and improve quality of life. Further, pain relief can be achieved through drugs, therapies and alternative therapies [3]. Traditional medicine has strong cultural roots, and many plants are used to treat pain. On the other hand, this ancestral knowledge can be the source of inspiration for finding numerous active ingredients and accordingly enabling therapeutic innovation in the management of pain and inflammation. In conclusion, many aromatic plant species have essential oils with antinociceptive activity. As a general rule, animal studies are essential for research aimed at understanding complex questions in relation to disease progression or other biological mechanisms. Since the use of human subjects in research is ethically inconceivable, the need to use animal subjects in the initial research process is essential despite the complexity of assessing pain and its manifestations, as well as measuring pain effects of potential analgesic molecules. It remains for the researcher to choose the best tests to obtain answers and identify new effective compounds with analgesic and anti-inflammatory potential.

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