**Analgesic and Anti-Inflammatory Effects of Methanolic Extracts of Immature Flowers Achillea wilhelmsii C. Koch in Mice (Balb/c)**

Mahdis Sarshogh¹, Ramesh Monajemi²*, Leila Amjad³

1- M.Sc student, Department of Biology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran
2*- Corresponding author, Assistant Professor, Department of Biology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran
3- Assistant Professor, Department of Biology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran

**Abstract:** Considering the role of pain and inflammation in delaying in the treatment of diseases and lack of a proper treatment without any side effects for the pain and inflammation using chemical drugs, and also considering anti-inflammatory and analgesic properties of Achillea wilhelmsii C.Koch in traditional medicine, this study was carried out to study the anti-inflammatory and analgesic effects of methanolic extract of Achillea wilhelmsii C. Koch Immature Flowers on mice. In this study, analgesic effects were studied using formalin test and anti-inflammatory effects were studied using xylene test. In each test, 30 mice which weight ranged from 20 to 25 grams were randomly divided into 5 groups each including 6 mice, namely the negative control group (receives Phosphate buffered saline and Tween 80%), the positive control group in formalin test (receives 10 mg/Kg morphine), the positive control group in xylene test (receives 15 mg/Kg Dexamethasone), and 3 experimental groups. Different concentrations of the methanolic extract (100, 200, 300 mg/Kg) were intraperitoneally injected into the mice of the experimental groups. The resulted data were analyzed by statistical tests. Results showed that compared to the negative control group, all different concentrations of the methanolic extract of Achillea wilhelmsii C.Koch significantly reduced acute and chronic pain in formalin test, and compared to the negative control group, just in the highest concentration (300 mg/Kg) the anti-inflammatory effect of the methanolic extract was significant and they were not significantly different compared to the positive control group. considering the findings, methanolic extract of Achillea wilhelmsii C.Koch is effective in reducing inflammation and pain.

**Keywords:** Achillea wilhelmsii C.Koch, Analgesic, Anti-Inflammatory, Methanolic extract, mice.

**Introduction**

Pain is one of the most common problems which human has been involved with from ancient times and has tried to find ways to avoid it. Pain appears in acute and chronic forms which can prohibit daily activities as a limiting factor in both forms. Acute pain is created by fast injures and disappears by eliminating the causing factor. Chronic pain is long time and is due to long-term injures and lasts until remaining the reason (Taherian et al. 2007).

Inflammation is an immune response which includes various complicated processes such as increase the permeability or vasodilatation, exudation of substances such as plasma proteins of blood vessels and the entry of leukocytes into the inflamed area leading to localized accumulation of plasma liquid and blood cells. In this defense mechanism, complicated events and mediator processes are involved to induce, maintain or exacerbate inflammatory reactions. Therefore, anti-inflammatory factors are effective in curing pathological reactions (Eidi et al. 2009).

Analgesic and anti-inflammatory chemical drugs are divided into narcotic analgesics and nonsteroidal analgesic, anti-inflammatory drugs (Khalili et al. 2011). Many narcotic drugs exist which their side effects Threaten patients during using period (BrownGuy & NeherJonas. 2010).

Achillea wilhelmsii C. Koch (Asteraceae) is widely found in different parts of Iran (Amjad et al. 2012). Since active ingredients of herbal drugs are accompanied and balanced with other matters, are not accumulated in the body and don’t have side effects, hence, have significant advantages compared to chemical drugs (Mohammadi-Sichani et al. 2011). Among medicinal plants, Achillea wilhelmsii is considered because of its extensive health benefits. It has been used for thousands years to treat various diseases especially infection diseases. In Greece stories, Achelous was placing pressed leaves of Achillea wilhelmsii on scars to prevent soldiers bleeding. This effect is confirmed now by scientist (Mirheydar. 1994).

This plant has been used in traditional medicine for reinforcing the stomach and carminative, as a sedative for gastric pains. Herbal tea of this plant is useful in treating indigestion with a bloated feeling and increased acid stomach (Giorgi et al. 2009). Achillea wilhelmsii is useful for nervous system and the heart, including the treatment of general fatigue, weakness, heart and neurological diseases such as neurasthenia, hysteria and epilepsy, due to tannins and bitter and aromatic substances. Essence of this plant is also effective in the treatment of neuralgia and rheumatism. Anti-inflammatory properties of extant flavonoids in Achillea wilhelmsii are because of its inhibitory effect on metabolism of...
arachidonic acid (Arzi & Akhavan, 2001). Achillea wilhelmsii plant has potential anti-oxidant properties due to the presence of flavonoids such as apigenin, luteolin, verotin and phenolic compounds (Giorgi et al. 2009). Methanol extract of this plant’s flower has anti-fungus properties (Amjad et al. 2012).

Considering the role of pain and inflammation in postnong diseases treatment and lack of chemical treatment without side effects and in view of Achillea wilhelmsii properties in treatment of swelling and pain in traditional medicine and also since there is no research about this plant, current study was carried out to investigate analgesic, anti-inflammatoriy effects of methanol extract of immature flowers of Achillea wilhelmsii on Syrian mice.

Materials and methods
Preparation of methanol extract
Wild Achillea wilhelmsii plants were collected from Chaharmahal and Bakhtiari province and confirmed by herbarium department of Research Institute of Forests and Rangelands. Immature flowers were separated and aerial parts were dried in room temperature for three days and was powdered using mill. Maceration method was used to extract preparation. 30g of prepared powder was dissolved in 250 ml of solvent (80cc methanol plus 20cc of twice distilled water). herbal solution containing erlen was placed on a shaker for 72 hours at room temperature. After that, solution was filtered, herbal part was separated, and methanol extract was poured in plates to dry.

Animals
Syrian little laboratory mice (Balb/C) from weight range of 20-25g were prepared and kept in a animals room at 22±2°c and 12:12 hours photoperiod. All Principles of Neuroscience Research Center Ethics Committee relating to the use of laboratory animals were considered such as free access to food and water, preventing surgery pain, using standard methods of killing animals, no additional animals in each treatment group. Mice were kept one week before experiment to adapt to environment and were kept in polycarbonate cages with stainless steel mesh lid.

Formalin test
Formalin test devised by Dubuisson & Dennis which is a model for pain assessment was used in this study. Five groups with six mice in each group were selected. Mice were placed in pain box several times 15 minutes each time to adapt to experiment situation.

Pain box in this study was a six part box made of glass with a wooden lid. This box was placed on a wooden chair which a camera was placed beneath it to filming mice activities. Positive control group received 10 mg/kg of morphine. Negative control group was injected phosphate buffered saline and Tween 80 % (with a ratio of 1:4); experimental groups received methanol extract of immature flowers in 100, 200 and 300 mg/kg of animal weight doses. Rapidly after injections, animal was placed again in pain box for 30 minutes.

After this time, 0.02 ml of formalin (Qatranshimi) 2.5 % was injected under the right paw subcutaneously. Mouse was returned to test box and was filmed for one hour in a quiet place and responses of animal to pain stimulus were recorded for one hour (Vahidi et al. 2001). Every 15 seconds, responses were scored from zero to four according to Dobson and Denis method. First five minutes was considered as first phase (acute pain), and 15 to 60 minutes as second phase (chronic phase) of formalin test. Data were recorded as 12 blocks of five minutes. Data recorded for 60 minutes after formalin injection (Nasri .2012).

Xylene test
To evaluate inflammation, ear edema test was used. At first, edema was created in ear using an external inflammation-causing material (xylene). After that, the extract ability to inhibit edema was evaluated. Five groups with six mice in each group were evaluated. Negative control group was injected 0.2 ml of phosphate buffered saline and Tween 80 %. Positive control group received 15 mg/kg of dexamethasone. Experimental groups received methanol extract of immature flowers in 100, 200 and 300 mg/kg of animal weight doses dissolved in phosphate buffered saline and Tween 80 %. all injections were done intraperitoneal. After 15 minutes, 0.03ml of pure xylene was injected subcutaneously on the dorsal surface of the right ear, after a mild anesthesia with ether. Left ear was considered as control. two hours later, animal was killed (Hosseinzadeh et al. 2000). Then, both ears were cut and 7 mm slices of ears were separated (from injecting places), weighed and weight difference was calculated. Higher weight difference shows higher inflammation (Hoodgar et al. 2011).

Statistical analysis
Obtained data were analyzed using SPSS 18. Results were presented as average± statistical deviation. One-way analysis of various, LSD test and Duncan test were used at five percent probability level.

Results
In this study which was carried out using formalin test, pain response was evaluated in two stages: 0-5 minutes and 15-60 minutes after formalin injection.

Variance analysis test was used to investigate analgesic effects of Achillea wilhelmsii methanol extract. It showed that all groups did not have equal pain responses.
Injection of methanol extract in all doses reduced acute pain in proportion to negative control (P<0.001). This effect was more obvious in 200 mg/kg dose (graph 1).

Graph 1. Comparison of acute pain intensity in treatment groups (***: p<0.001)

Also, all doses of extract reduced motor response of chronic pain. The effect was more obvious in 100 and 300 mg/kg doses (graph 2).

Graph 2. Comparison of chronic pain intensity in treatment groups (***: P<0.001, **: P<0.01)

Difference between ears weight was not significant in 100 and 200 mg/kg doses in proportion to negative control and only 300mg/kg group reduced the inflammation significantly (p<0.05) (graph 3).
Discussion

Formalin test has been used as an experimental model of neurogenic inflammation pains. Pain caused by formalin is different from acute pains. This type of pain is caused by tissue damages, inflammation, and sensitivity of sensory fibers and pain-related behaviors are very similar to common human pains (Dashti-Rahmatabadi et al. 2009).

One of formalin test specifics is that animal shows two responses to pain which are originated apparently from two different mechanisms. First stage appears immediately after formalin injection, and lasts for three to five minutes. Apparently this pain is due to chemical stimulation of pain receptors. Second stage starts 15 to 20 minutes after injection and animals begins again to lick the soles of its feet which lasts about 20 to 30 minutes. It seems that peripheral inflammatory processes are involved in this stage. In this test, two obvious pain stages are observed: in first phase PM matter and bradykinin and in second phase Histamine, serotonin, bradykinin, and prostaglandins play role which this can itself show inflammatory aspect of second stage (Tjolsen.1991).

First pain stage is in accordance with acute or neurogenic pain that is sensitive to drugs which act through opioid system. Second stage is the same inflammatory pain which is controlled by both opioids and nonsteroidal drugs. Substance such as morphine which act basically as central analgesia and control both stages, while drugs such as aspirin act via peripheral nervous system, and inhibit only the second stage pain. This action is done either by blocking the receptors or by inhibiting production or release of mediators (Rosland et al. 1990).

Since studied plant reduced pain in both phases (early and late), it shows that central and peripheral mechanisms are involved in analgesia. The reason of controlling pain in chronic phases by anti-inflammatory drugs is blocking prostaglandins synthesis (Zeashan et al. 2009).

Pain control in second phase can show that the extract enforces its analgesic effects probably by inhibiting the synthesis or release of mediators of inflammation. Researches have shown that flavonoids have considerable analgesic effects which cause controlling effect on release of arachidonic acid and bradykinin (Mada et al. 2009).

Since Achillea wilhelmsii is a source of flavonoids, it can be an analgesic treatment. Flavonoids are from nitric oxide synthase enzyme inhibitors which are increased following formalin injection (Toker et al. 2004). Since nitric oxide can be pain mediator, reducing it leads to analgesic activity (Mehmet et al. 2003). also, research reduces have shown that flavonoids reduced pain via opioid and adrenergic system (Anjaneyulu & Copra. 2003). Studies of Amjad et al. (2016), showed that immature flowers of Achillea wilhelmsii have the highest flavonoid compounds and anti-oxidant.

Some of inflammation probable mechanism can be identified, in inflammation test which was done using xylene. Xylene is a substance which causes neurogenic inflammation (Rotelli et al. 2003).It means that a group of specific compounds are released from sensory neurons, these substances lead to dilution of blood vessels and may increase vascular permeability and leukocyte influx (Vogel. 2002).

In cellular mechanisms of neurogenic inflammation, substances are released from the end of capsaicin-sensitive sensory neurons which include neuropeptides and P matter. It is reported that capsaicin-like molecules affect primary afferent neurons of thin C fibers which are connected to discrete sensory receptors. Neurogenic inflammation due to release of vasoactive peptide transmitters such as P matter and calcitonin gene-related peptide from peripheral terminals of afferent neurons fibers. These materials are main initiator of inflammation (Richardson et al. 2002).

Controlling effects of flavonoids on acute and chronic inflammation is because of affecting signaling path including activity of kappa B, neuclear factor and phosphorylation of MAP kinase. Furthermore, flavonoids reduce...
accumulation of floating lipids which are necessary for signaling pain phenomenon. Therefore, flavonoids reduce acute and chronic inflammation by inhibiting aggregation of receptor and signaling cascade (Nasri et al. 2008). On the other hand, anti-inflammatory effects of flavonoids are enforced by inhibiting the production of inflammatory cytokines such as tumor necrosis factor from activated macrophages in inflammation. These pro-inflammatory materials increase prostaglandins synthesis. (Toker et al. 2004)

Tekieh et al. in a study on analgesic and anti-inflammatory effects of Achillea wilhelmsii injected defatted methanol extract in 50, 100, and 200mg/kg doses and announced that volume of the foot reduced during short-term treatment (6 days) dose dependently in third and sixth days. 50 and 100mg/kg doses could not reduce foot volume significantly in third and sixth days in proportion to injection of complete Freund's adjuvant (positive control) whereas 200mg/kg reduced it significantly. The extract was effective in dose dependent reduction of inflammatory symptoms especially hyperalgesia, edema, and IL-6 serum during short term inflammation induced by complete Freund's adjuvant. Methanol extract of this plant -because of having flavonoids- enforces appropriate anti-inflammatory and analgesic effects non-opioid analgesic pathway by inhibiting the release or synthesis of inflammatory mediators responsible for causing pain. Methanol extract of immature flowers of achilla wilhelmsii had significant analgesic effects in all doses; therefore, in accordance with results of Tekieh et al., probably flavonoids which are natural polyphenol compounds of Achillea wilhelmsii are the reason of its analgesic, anti-inflammatory effects.

Conclusion
Considering the results of this study, methanol extract of Achillea wilhelmsii had obvious analgesic, anti-inflammatory effects, wider use of these plants as a medication is expected by using the results of this study with further researches.

References