

Anti-inflammatory and analgesic effect of an alkaloid-fixed oil mix from *Linum usitatissimum* seeds *in vivo*

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Abstract

Inflammation is an immune response to chemical, physical or biological aggression. The comprehension of the several pro-inflammatory and pro-resolving process may lead to the development of new functional drugs instead of the conventional ones. However, Inflammation reaction is conventionally treated by steroidal and nonsteroidal anti-inflammatory drugs. These medications may be useless because of their side effects. In this study, we aim to evaluate the anti-inflammatory and analgesic effect of alkaloid /fixed oil mixture (AO) from flaxseeds *in-vivo*. Three doses of AO mix (50, 100 and 200 mg/kg) were used in several anti-inflammatory and analgesic tests. The obtained results had shown that the AO mix presents a potential anti-inflammatory and analgesic activities. These results highlight the therapeutic potential of alkaloids and fixed oils combination. Additionally, to other research, the present study supports the anti-inflammatory and analgesic effects of flax seeds with an original contribution by combining alkaloids and fixed oil from *Linum usitatissimum*.

Keywords: Inflammation, anti-inflammatory, analgesic, *Linum usitatissimum*, Alkaloid, fixed Oil.

Introduction

Inflammation is an immune response to chemical, physical or biological aggression; it is characterized by several events resulting in the formation of edema by an inflammatory mediators release such as bradykinin, serotonin, histamine, leukotrienes and prostaglandins (Macedo et al., 2019). The understanding of how the inflammatory process is activated and the comprehension of the high plasma expression of pro-inflammatory cytokines like interleukine (IL-1), IL-6 and tumor and necrosis factor α (TNF- α) may lead to the development of strategies to block or reduce the inflammation response (Ward and Lentsch, 1999). Inflammatory reaction can be devised in 3 phases: Silent phase, vascular phase and cellular phase. This reaction leads to a sensory response including pain, hyperalgesia and allodynia. Any inflammatory process inappropriate regulation may lead to significant tissue dysfunctions which are translated into several inflammatory diseases such as arthritis, inflammatory bowel disease and asthma (Vergnolle, 2003).

The inflammation is naturally resolved with cellular and molecular mediators, the inflammatory response is characterized by increased blood flow, capillary dilatation and leucocytes infiltration especially neutrophils which plays a key role in the inflammation process. However, during the inflammation resolution we can observe the depletion of neutrophils from the inflamed site through a programmed process that is regulated at multiple levels. In addition, several cellular and molecular process occur when inflammation resolution process is activated like: the counter regulation of chemokines and cytokines, the switching off of signalling pathways associated with leukocytes survival, the reprogramming of macrophages from classically activated to alternatively activated cells and finally the initiation of healing process (Sugimoto et al., 2016).

The comprehension of the several pro-inflammatory and pro-resolving process may lead to the development of new functional drugs instead of the conventional ones. Inflammation reaction is conventionally treated by steroidal and nonsteroidal anti-inflammatory drugs (SAIDs, NSAIDs) to relief pain and inflammation. However, these medications may be useless because of their side effects.

New researches are nowadays recommending the use of medicinal plants that have a higher interest and present a minimum of side effects (Bribi et al., 2016; Rafieian-kopaei et al., 2017a).

Linum usitatissimum that belong to Lineaceae family is an annual plant known to be used in food and textile industries. The flax seeds oil is also known, due to its high content of polyunsaturated fatty acids, to have a wide range of health benefits. (De Santana Lopes et al., 2018; Kaithwas et al., 2011). On the basis of previous work, in which *Linum usitatissimum* had been reported as a plant with biological effects on acute and chronic arthritic albinos rat (Kaithwas and Majumdar, 2010b), antibacterial activities of hydrolysed lipid against *S. aureus* resistant to antibiotics (McDonald et al., 1981) and an antiulcer activity of flaxseed oil in animal model (Kaithwas and Majumdar, 2010a), these pharmacological activities shows the high interest of this plant (Kaithwas et al., 2011). In this study, we aim to evaluate the anti-inflammatory and analgesic effect of alkaloid /fixed oil mixture (AO mix) from flaxseeds *in vivo*

Material and methods

Drugs and chemicals

All substances were purchased from Sigma-Aldrich Chemical (Madrid-Spain), unless otherwise stated. The test substances were dissolved in distilled water and prepared fresh daily for administration to the animals.

Animals

Healthy male albino NMRI mice (26-32 g) from the Laboratory Animal Service of the University of Bejaia (Algeria), were maintained under standard laboratory conditions with free access to tap water and food. The experimental protocol was carried out in accordance with the 'Guide for the Care and Use of Laboratory Animals as promulgated by the National Institute of Health, and the protocol approved by the local Ethics Committee of the laboratory of PBVE, University of Bejaia, (Ref. No. CE-LBVE-2017-107).

Alkaloids extraction

In the present study we used *Linum usitatissimum* seeds (flax seeds) from commercial source at Bejaia city. The alkaloid extraction were performed following Soušek protocol (Soušek et al., 1999). Briefly, the flaxseeds powder was extracted with methanol for 8 h in a Soxhlet apparatus, and then evaporated under reduced pressure, acidified with 2.5% HCL to pH 1-2 and filtered, and stored at room temperature overnight. The aqueous acid solution was adjusted to pH=9.5 with concentrated ammonium hydroxide and extracted with dichloromethane. The extracts were evaporated to afford a crude extract of total alkaloids. After evaporation the alkaloid extract of *Linum usitatissimum* (ALU) obtained was stored at 4°C until use.

Fixed oils extraction

Flaxseeds were washed and grinded then macerated in methanol under agitation, the fixed oils extraction was a solid-liquid extraction. In brief, 500 ml methanol was added to 50 g of flax seed powder, after maceration we performed a phases separation to recover the fixed oil after evaporation (Danlami et al., 2014). Due to miscibility of fixed oil we dissolved it in tween 20.

Mouse ear edema induced by xylene

In a first time, edema induced by the topical application of chemical irritants was measured as an increase in ear thickness, the chemical irritant (30 µl of Xylene) was pipetted directly into the ears of the mice. Ear thickness was assessed with an electronic digital micrometre (Starrett Series 734) applied near the tip of the ear distal to the cartilaginous ridges; the difference in thickness between the basal measurement and after challenge was calculated (Macedo et al., 2019). Treatment was applied with oral administration of AO mix (50, 100 or 200 mg/kg), and diclofenac (5 mg/kg) or local application of AO mix (0.25, 0.5 or 1 mg/kg) and diclofenac (0.5 mg/kg)

In a second time, the test of xylene-induced ear edema in mice was based on the reported method (Nunez-Guillen et al., 1997; Akindele and Adeyemi, 2007). Briefly, thirty minutes after oral administration of AO mix (50, 100 or 200 mg/kg), and diclofenac (5 mg/kg), an edema was induced in

the right ear by topical application of 30 µl xylene applied near the tip of the ear distal to the cartilaginous ridges. After 15 min, the mice were sacrificed by cervical dislocation. Circular sections of 7 mm diameter were removed from each ear using a cork borer and weighted. The edema degree was responded with the difference in weight between treated and no-treated ear from each mouse to evaluate the effect of AO mix. The anti-inflammatory effect was expressed as percentage of the inhibition of the oedema (PI). This percentage was calculated using the following formula:

$$PI = \frac{\text{Difference in ear weight, control} - \text{Difference in ear weight, treated}}{\text{Difference in ear weight, control}} \times 100.$$

Acetic acid-induced writhing response

The writhing test was carried out as previously described (Koster et al., 1959). Mice were randomly assigned to five groups, and after an overnight fasting period, they were pre-treated with AO mix (50, 100 or 200 mg/kg, p.o.), diclofenac (100 mg/kg, p.o.), or distilled water (control group, p.o.), 60 min before the acetic acid injection (10 mL/kg body weight, i.p.). Immediately after the injection of acetic acid, each animal was placed in a transparent plastic observation chamber. Five minutes after the administration of 1% acetic acid^{v/v}, the number of writhes and stretching movements of each mouse was counted for a period of 15 min. The percentage of inhibition of writhing was calculated and compared with the control group using the expression: Inhibition (%) = $\frac{WC-WT}{WC} \times 100$; WC: mean of writhing (control); WT: mean of writhing (test). (Bribi et al., 2016)

Formalin-induced Licking paw

The formalin test was performed as previously reported (Hunskar and Hole, 1987; Tjølsen et al., 1992). Briefly, overnight fasted mice were divided into five groups, which received distilled water (10 mL/kg, p.o.), AO mix (50, 100 or mg/kg, p.o.), or diclofenac (100 mg/kg, p.o.) 1 h before formalin injection (20 µL of 1% formalin) under the plantar surface of the right hind paw. The mice were then placed in a transparent box for observation, and the time spent licking the injected paw was measured and considered as an indication of inflammatory-associated pain. The first phase of the nociceptive response normally peaks 0–5 min after injection and the second phase 15–30 min after.

Tail-immersion test

The tail immersion test was used to evaluate thermal-induced pain following Swell and Spincer (Sewell and Spencer, 1976). Briefly, each mouse was placed in a holder with its tail protruding. The tail was placed in a hot water bath at 50±2 °C until the tail whipped or a flinch of body occurred. A cut off time of 15 seconds was imposed. Mice were treated for control test and Mix (50, 100 and 200 mg/kg) analgesic effect. The anti-nociceptive response was calculated as follow:

$$\% \text{ anti-nociception} = \frac{T-C}{15-C} \times 100$$

Where C and T represent the tail whip reaction time in seconds prior to narcotic injection (C) and at the peak time (T) after the day injection (Statile et al., 1988).

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). The statistical analysis of all the observations was carried out using one-way ANOVA followed by multiple comparison test of *Dunnnett's*, where necessary. A difference of p<0.05 was considered as significant compared with the negative control (treated with vehicle).

Results and discussion

Anti-inflammatory activity

Xylene induced-ear edema

The evaluation of anti-inflammatory effect of the alkaloids-fixed oils mix (AO) extract from *Linum usitatissimum* was performed through a xylene induced-ear oedema following different measurement and treatment modalities. The oral and local administrations of (AO) of *Linum usitatissimum* produced a marked analgesic and anti-inflammatory effects in models of pain and

inflammation. The results of inhibition of the xylene-induced ear edema in mice are presented as means \pm SEM.

In a first place, we realized a measure of ear thickness with two treatment administration ways; local administration (AO mix 0.25, 0.5 and 1 mg/kg, diclofenac 0.5 mg/kg). The results are presented in (Figure 1: A), these results show a significant inflammation inhibition by the different AO mix doses (0.25, 0.5 and 1 mg/kg) with respectively 42.5%, 51.8% and 69.98%. Indeed, AO mix shown a dose dependant anti-inflammatory that was close to 5 mg/kg diclofenac effect (75.09%) with a 1 mg/kg AO mix dose.

Then, we performed an oral administration of 3 doses of AO mix (50, 100 and 200 mg/kg), and diclofenac (5 mg/kg), the results are presented in (Figure 1: B). In this case, AO mix doses and diclofenac also shown a significant reduction of ear edema ($***P<0.001$). The 5mg/kg diclofenac dose has an inflammatory inhibition percentage as high as the 200 mg/kg AO mix dose with 74.25% and 80.83% respectively. The differences between the results of the two administration modalities may be due to the pharmacological proprieties of the active molecules among the AO mix of flax seeds, which leads to realise more pharmacological and toxicological investigations.

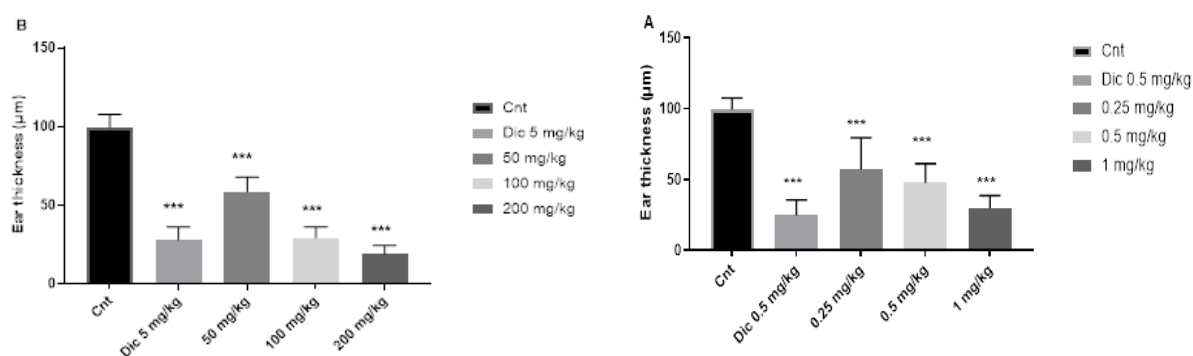


Figure 1. Anti-inflammatory effect of AO Mix on mouse ear oedema induced by Xylene. (A): PI % with local administration “per se”, (B): Ear thickness with local administration “per se”, (C): PI % with oral administration “per os”, (D): Ear thickness with oral administration “per os”.

In a second place, we investigate the difference between ears weight (Control ear – treated ear). The results presented in Figure 2 shows low PI (%) for the 50 mg/kg dose of AO mix and no statistical significance. However, 100 mg/kg and 200 mg/kg presented a better effect; the 200 mg/kg dose of AO mix was even higher than the 5 mg/kg diclofenac dose with 62.9 % against 47.26%.

Xylene is one of common pollutants in the plastic, chemical and leather industries. Skin contact with this chemical substance may result in local disorders such as irritant dermatitis or allergic dermatitis (Sasseville, 2008). An investigation about the role of TRPA1 receptors in skin inflammation induced by volatile chemical irritant shows that Xylene may promote vascular reaction mediated by TRPA 1 receptor signalling while this channel is expressed in many non-neuronal cell such as mast cell, keratinocytes and melanocytes. In addition, this study has also established that TRPV1 may work in a synergic manner with TRPA1 to mediate the cutaneous inflammatory response caused by xylene (Norões et al., 2019).

Another study about effect of flaxseed fixed oil against distinct phases of inflammation had shown that *L.usitatissimum* fixed oil significantly reduced the peritoneal vascular permeability, indicating the suppression of the vascular response during the acute inflammation. Also, flaxseed fixed oil inhibited the leucocytes migration (Kaithwas and Majumdar, 2013). Furthermore, it has been established that flaxseed oil had a beneficial effect in diet which might be due to the modulation of omega-6 availability and immunological homeostasis (Singh et al., 2012). In the light of this investigation, AO mix could combine additionally or synergically the immunological modulation of each alkaloids and fixed oil from *L.usitatissimum*.

Taken together, these results show the anti-inflammatory effect of AO mix in the Xylene induced ear oedema. Several studies used this acute inflammation model induced by Xylene for its capacity to promote inflammation especially through enzymatic pathway such as phospholipase A₂(Bribi et al., 2016; Macedo et al., 2019)

The several comparative graphs in Figure 3(A and B) that shows the administration modalities (local or oral) and the PI (%) measurement, leads to the same results highlighting the regular anti-inflammatory effect of AO mix which seems to be a dose-dependent effect.

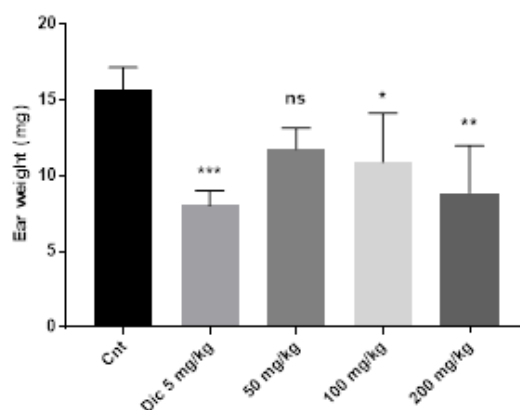


Fig 2. Anti-inflammatory effect of AO mix and diclofenac on Xylene induced ear oedema, (A): PI (%) calculated from weight difference between control and treated ear. (B): Weight differences between control and treated ear (mg). ***P<0.001, **P<0.01, *P<0.05, ns P>0.05 vs control group (cnt).

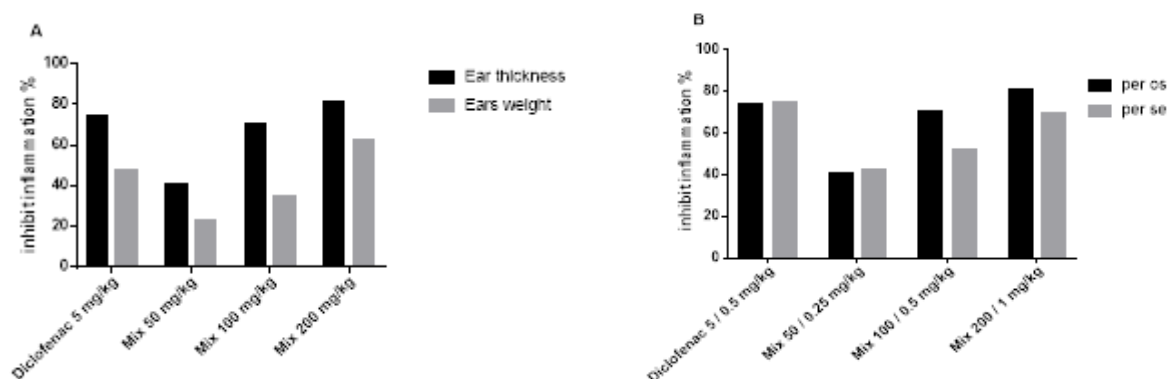


Figure 3 Treatment and measurement modalities dependent Anti-inflammatory effect of AO mix and Diclofenac, (A): compare between PI (%) of different oedema measurement (Ear thickness and Ears weight), (B); compare between PI(%) of different application modalities (per os and per se).

The results presented in Figure 4(A) shows a significant analgesic effect of the Ao mix (P<0.001 vs the control group). The Figure 5 (B) shows a writhing response inhibition percentage higher than 80% for 50, 100 and 200 mg/kg doses of AO mix.

Analgesic activity

Acetic acid-induced writhing response

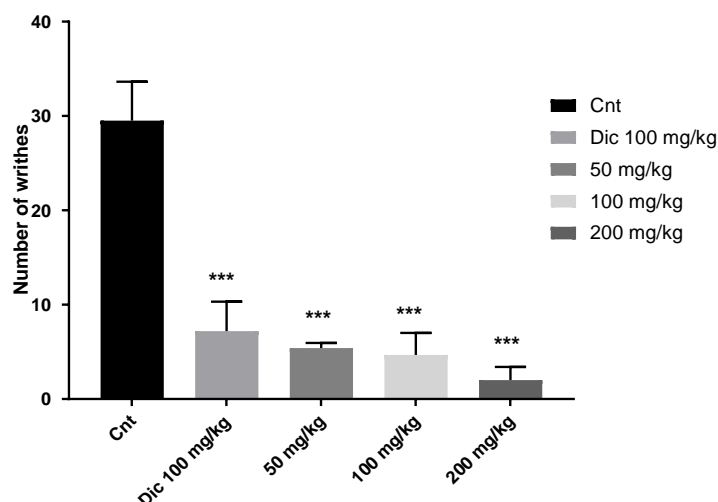


Figure 4 Analgesic effect of AO mix on mice acetic acid-induced writhing response. (A): Number of writhes, (B): percentage of writhing response inhibition. *** $P < 0.001$ Vs control group (cnt).

The acetic acid injection in the peritoneal cavity can induce an inflammatory response followed by a sensorial one, which leads to the release of several inflammatory and hyperalgesia mediators that promote the exciting of nerves extremities translated into the symptomatic writhing response (Bribi et al., 2016). Local tissue injury prompts the release of chemical mediators (potassium, hydrogen ions, and bradykinin) and inflammatory mediators as prostaglandin E₂ (PGE₂) from inflammatory cells. These substances directly activate nerve endings and trigger the release of algogenic mediators (for example, histamine, serotonin (5-HT), nerve growth factor (NGF), and prostanoids) from other cells and afferent nerves (Bribi, 2018).

Licking paw test

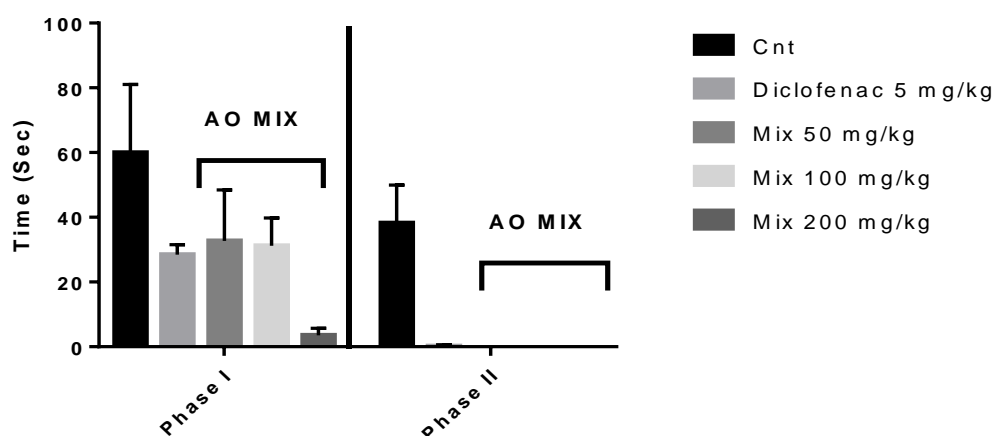


Figure 5. Analgesic effect of AO mix (50, 100 and 200 mg/kg) on formalin-induced licking paw test.

The formalin induced licking paw test is a communally used analgesic test, divided into two successive phases the first one shows the anti-nociceptive response while the second one shows the anti-inflammatory response. The AO mix (50, 100 and 200mg/kg) effect on the first phase (anti-inflammatory phase) approve the anti-inflammatory effect of AO mix previously evaluated (Figure 1, 2 and 3), translated in a significant reduction of licking time compare to the control group. The 200 mg/kg dose of AO mix had reveal a significant anti-inflammatory effect on the first phase even compared to 5

mg/kg diclofenac effect. In the second phase (anti-nociceptive), the three doses of AO mix (50, 100 and 200 mg/kg) had shown a significant analgesic effect with no licking time.

The tail immersion test

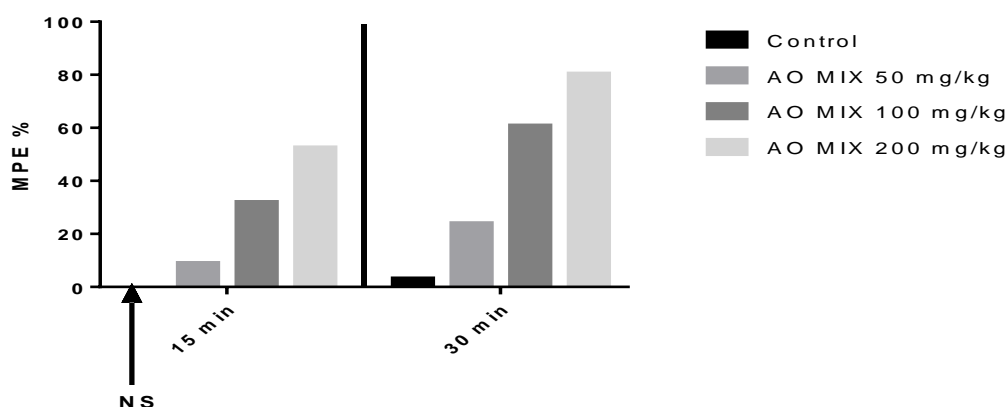


Figure 6. Anti-nociceptive effect of Ao Mix (50,100 and 200 mg/kg) on Tail Immersion Test

The tail immersion test used in this study was performed to evaluate the central nociceptive effect of AO Mix. The results show that Ao mix had a dose dependent analgesic effect with the high MPE % at the highest AO Mix dose (200 mg/kg) during the two phases of the test (0-15 and 0-30).

Taken together, these results highlight the analgesic effect of *linumusatissimum* on the different analgesic tests performed in this study. In fact, both formalin induced paw licking test and tail immersion test coordinate with the acetic acid-induced writhing response results showing that AO mix had peripheral and central anti-nociceptive effect, since assayed analgesic tests in this investigation evaluate peripheral and central analgesic effect. In previous studies, anti-inflammatory and analgesic effect of effective compounds in nociception have been attributed to some alkaloids, flavonoids, organic acids or caffeic acid derivatives (Rafieian-kopaei et al., 2017b).

It appears that the combination of alkaloids and organic acid in the fixed oil have an additional or synergic analgesic effect. Substances characterisation is needed to determinate which compound in the AO Mix is responsible of either analgesic or anti-inflammatory activity or even the two activities at the same time, considering that pain and inflammation are much related possess.

Conclusion

The alkaloids/fixed oil mixture from *Linumusatissimum* (Flax seeds) shows a significant anti-inflammatory and analgesic activity *in-vivo*, these results highlight the therapeutic potential of alkaloids and fixed oils combination which may combine different actives compounds acting with different mechanisms in an additional or synergical ways. Additionally, to other research, the present study supports the anti-inflammatory and analgesic effects of flax seeds with an original contribution by combining alkaloids and fixed oil from *Linumusatissimum*.

Author's Contributions

Riad Fehat, Mohamed sofiane Merakeb and Nouredine Brihi performed the experiments and contributed to the acquisition and analysis of data. Riad Ferhat, Nouredine Brihi and Betitra Yanat contributed to the analysis and interpretation of data, designed the experiments and wrote the manuscript.

Conflicts of interest

All authors declare that there is no conflict of interest

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