



ANALGESIC, ANTI-INFLAMMATORY AND ANTIDEPRESSANT ACTIVITIES OF TRITERPENE FROM MEIIOCARPIDIUM LEPIDOTUM (ANNONACEAE) BARK

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ABSTRACT

The traditional phytomedicine: *Meiocarpidium lepidotum* (Oliv.) Engl. & Diels (Annonaceae) has been used in Fang community to treat illness. This study was designed to verify the antinociceptive, antidepressant, anti-inflammatory and acute toxicity of *Meiocarpidium lepidotum* (ML) extract in rats and mice. The crude aqueous extract of plant containing triterpene was used in different studies. Phytochemical qualitative analysis was performed using classical and UV methods. The analgesic activity was assessed by employing different pain models, such as tail flick tests for central analgesia, acetic acid-induced writhing (peripheral analgesic model), carrageenan-induced hyperalgesia (inflammation model) in mice. In rats, an antidepressant activity was assessed by swimming test. Our study demonstrated that the crude aqueous extract of ML containing triterpene was not toxic, was capable of preventing nociception, inflammation and depression at a concentration of nearly 1 mg/kg of bodyweight. In mice, ML induced reduction of swelling of the edema paws. This anti-inflammatory effect is similar to the one obtained with 150 mg of acetylsalicylic acid (ASA) and 10 mg of indomethacin for 3 hours. It significantly reduces after 4 hours ($p < 0.001$). The analgesic effect of 1 mg/kg of ML was similar to the effect of 0.1 mg/kg of morphine (Mph) in tail-flick tests ($p < 0.001$) and that of 150 mg of ASA in acetic acid-induced writhing test. In addition, this study showed that 1 mg/kg of ML exhibited antidepressant effect similar to the one obtained with 32 mg/kg of Fluoxetine ($p < 0.01$). To our knowledge, this is the first demonstration of the pronounced analgesic, anti-inflammatory and antidepressant effect of ML in vivo studies. This work validates the traditional use of ML in the Congo Basin forest as phytomedicine.

Key words: Carrageenan, tail flick, Antinociception, Anti-inflammation, Antidepressant, *Meiocarpidium lepidotum*, Congo Basin Forest.

INTRODUCTION

Meiocarpidium lepidotum (Oliv.) Engl. & Diels (Annonaceae) is one of the anti-dry cough and throat inflammation phytomedicine by the Fang ethnic community. The tree was found only in the Congo Basin forest, mainly in Cameroon, Gabon, Equatorial Guinea,

the Congo and Central African Republics. There is no available data of the traditional use of *Meiocarpidium lepidotum* (ML). Only the traditional utilization of wood to make various devices was reported (Leboeuf *et al.*, 1977b). A few phytochemical studies were done on ML compared to other Annonaceae. From root and stem bark of ML, two alkaloids were isolated, methoxyathero sperminine and N-oxy methoxyathero sperminine (Leboeuf *et al.*, 1977b), one triterpene, the *polycarpol* and lignan *meiocarpin* (Ngouela *et al.*, 2004). But the first phytochemical screening of aqueous extract showed that

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the extract did not contained flavonoid, lignan and alkaloids, suggesting that the biologic effect is the action of other chemical group.

The study was designed to verify the antinociceptive, antidepressant and anti-inflammatory effect and acute toxicity of ML in rats and mice. The result showed the analgesic antidepressant and anti-inflammatory effect of ML extract containing triterpene. This study validates the traditional use of ML against dry-cough and throat inflammation.

MATERIALS AND METHODS

Chemicals

All reagents were purchased and used for research use only: Fluoxetine, acetylsalicylic acid, indomethacin, morphin, acetic acid, λ -carrageenan (Sigma-Aldrich, St-Quentin Fallavier, France).

Plant resources and preparation of crude extract

The plant infusion was prepared according to the methods recommended traditionally, to be administered orally. The Stem bark of *Meiocarpidium lepidotum* (ML) was collected in July, 2012 by Prof Bruno ETO at Mekomo-Ambam, sub-region of Vallée du Ntem, in southern Cameroon, was identified by the National Herbarium of Yaoundé, Cameroon, and authenticated the voucher specimen (N° 26084). At the National Herbarium of Libreville, Gabon, the voucher specimen is Breteler, F.J. 13847 (WAG). Crude extract was obtained by maceration for 24 h of dried plant (500g) in boiling distilled water with stirring. The Macerate was then filtrated and lyophilized, obtaining 13% brown powder yield, and kept at 4°C until use.

Phytochemical study for primarily qualitative analysis of active principles

Qualitative analysis of LM for the presence of various medicinally important active phytochemicals was conducted using the same methods described earlier (Paech & Tracey, 1956).

Standard screening tests for detecting the presence or different chemical constituents like flavonoids, tannins, saponosids, alkaloids and anthracenosids were used.

Test for saponosids

With 0.5 g of the extract, 5 ml of distilled water was added in the test tube. The solution was shaken vigorously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil, and shaken vigorously. It was then observed for the formation of emulsion.

Test for flavonoids

Hydrolyze reaction: A diluted 20 ml HCL (2N) was added to a portion of an aqueous filtrate of the extract

(19 mg) and heated for 40 min before being stored at room temperature. A pink coloration indicates the presence of flavonoids.

Extraction of genuine: Dilute 20 ml of dichloromethane (CH_2Cl_2) to a portion of 20 ml of the extract. After shaking the mixture, its decantation induces two phases where the organic phase was lower (genuinely containing).

Characterization of flavonoids: The organic phase was used and shaken with 1 ml of dilute ammonia solution. A yellow colouration indicates the presence of flavonoids.

Test for terpenoids

Hydrolyze reaction: A diluted 20 ml HCL (2N) was added to a portion of an aqueous filtrate of the extract (19 mg) and heated for 45 min before being stored at room temperature. The presence of blue-violet florescence to red violet spots under visible light at UV 365 nm indicates the presence of terpenoids (Wagner *et al.*, 1984; Rabyah *et al.*, 2012).

Test for alkaloids

0.5 g of the extract was diluted to 10 ml of 2% aqueous sulphuric acid and filtered. To 5 ml of the filtered material, 3-4 drops of Mayer's reagent were added. The white precipitate indicates a positive reaction. In the second portion of the test, 3 or 4 drops of Dragendorff's reagent were added. The formation of a reddish brown or cream precipitate was regarded as a positive presence of alkaloids.

Test for tannins

About 0.5 g of the extract was boiled in 10 ml of water in a test tube, and then filtered. 10 ml of HCl (2N) were added and heated for 15 min. It was to observe for red colouration indicating the presence of tannins.

Test for anthracenosids

0.5 g of the extract with 10 ml of HCl (2N) were boiled together for 10 min, and filtered while hot. The filtered material was shaken with 10 ml of toluene. The toluene layer was piped into another test tube, and 1ml of the toluene phase was diluted with 1 ml of NaOH (1N). The resulting solution was observed for colour changes, to red purplish.

Animals

Wistar male rats, weighing 150–200g, and Swiss albino male mice, 20-30 g (Animal Laboratory of Medicine and Faculty of Pharmacy, Mohammed V Souissi University, Rabat, Morocco) were kept in cages under standard laboratory conditions, with tap water and standard rat chow ad libitum, in a 12-h/12-h light/dark cycle, at 21–23°C temperature. The study was conducted

in accordance with the accepted principles outlined in the "Guide for the Care and Use of Laboratory Animals", prepared by the National Academy of Sciences, and published by the National Institutes of Health. All efforts were made to minimize animal suffering and the number of animals used. Ethics approval was obtained from the Mohammed V-Souissi University, Faculty of Medicine and Pharmacy, Rabat, Morocco.

Acute Toxicity Study

The acute toxicology test in mice was conducted according to the OECD guidelines for testing of Chemicals (OECD, 2002). Male Swiss mice were randomly divided into two groups (10 mice per group). The mice were given ML (2g/kg) orally. The experimental mice were provided with forage and water ad libitum, and were kept under regular observation for 14 days for any mortality or changes in behaviour. The behavioural changes observed were hyperactivity, tremors, ataxia, convulsions, salivation, diarrhoea, lethargy, sleep, and coma.

Forced Swimming test

The Forced swimming (FS) test, described in (Porsolt *et al.*, 1977), was conducted. The procedure used was previously described by Detke *et al.* (Detke *et al.*, 1995). The animals (rats) were forced to swim inside a cylinder filled with water, and with no possibility for the animals to get out. The resulting anxiety produced a vigorous swimming activity, as attempts by the animals to escape from the cylinder, diving or climbing the walls, were unsuccessful. When the movements of the animals stopped, with the exception of those who kept on moving for survival (keeping the head above the water), the behaviour was considered immobile. This was classified as induced depression. On the first day (pre-session test), the rats were forced to swim for 15 minutes in an acrylic cylinder (30 cm diameter × 50 cm high) containing $23 \pm 1^\circ\text{C}$ water 40 cm deep. The water was changed after every swimming test to eliminate urine, excrement, and fur. After the swimming session, the rats were removed from the cylinder, dried with towels, and placed under a light bulb (32°C) for 15–30 minutes.

The test session: Animals received the dose of their respective drugs 1 hour(s) prior to the test session, and were tested under the same conditions for 6 minutes. The Immobility and number of times the rats stopped swimming (number of stops) were recorded. Immobility is referred to the absence of movement, with the body inclined forward, floating passively, and with the paws still. The swimming activity was recorded from the large forepaw movements displacing the body around the cylinder more than necessary, a performance to merely keep the head above the water. The Climbing was

recorded when the rat began vigorous movements with the forepaws in and out of the water, typically directed against the wall of the tank.

Acetic-Acid-Induced Writhing Response

The writhing test in mice was done according to the method of Koster *et al.* (Koster *et al.*, 1959). The writhes were induced by intraperitoneal injection of 1.2 % acetic acid (v/v, 0.15 ml/ 20 g body weight). There are three different doses (1, 10, and 100 mg/kg) of ML administered orally to each group of mice, 60min before chemical stimulus. Acetylsalicylic acid and physiologic solution, a positive and negative controls, were administered 30min prior to acetic acid injection. The number of muscular contractions was counted over a period of 5 min following the acetic acid injection. The data represented the total number of writhes observed during a 10 min period.

λ -Carrageenan-Induced Mouse Paw Edema

The anti-inflammatory activity of ML was determined by the λ -carrageenan-induced paw edema test in the hind paws of mice. The test was conducted according to the method of Vinegar *et al.* (Vinegar *et al.*, 1969). The basal volume of right hind paw was determined before the administration of any drug. Fifty microliter of 2.5% λ -carrageenan suspended in saline (0.9%) was injected into the plantar side of right hind paw. The paw volume was then measured at the 1st, 2nd, 3rd, and 4th hours after the injection using an LE 7500 plethysmometer (Panlab, Spain). The degree of swelling was evaluated by the delta volume (a-b), where "a" is the volume of right hind paw after the chemical treatment, and "b" the volume before the treatment, or $[(a-b)/b] \times 100$, expressed as a percentage. Indomethacin (10 mg/kg) was administered intra-peritoneally, whereas 150 mg of Acetylsalicylic acid (ASA) given orally 30 min before λ -carrageenan injection. LM (1, 10, and 100 mg/kg) was administered orally 60min before λ -carrageenan injection. The control was given an equal volume of saline.

Evaluation of central analgesic activity by Tail flick method

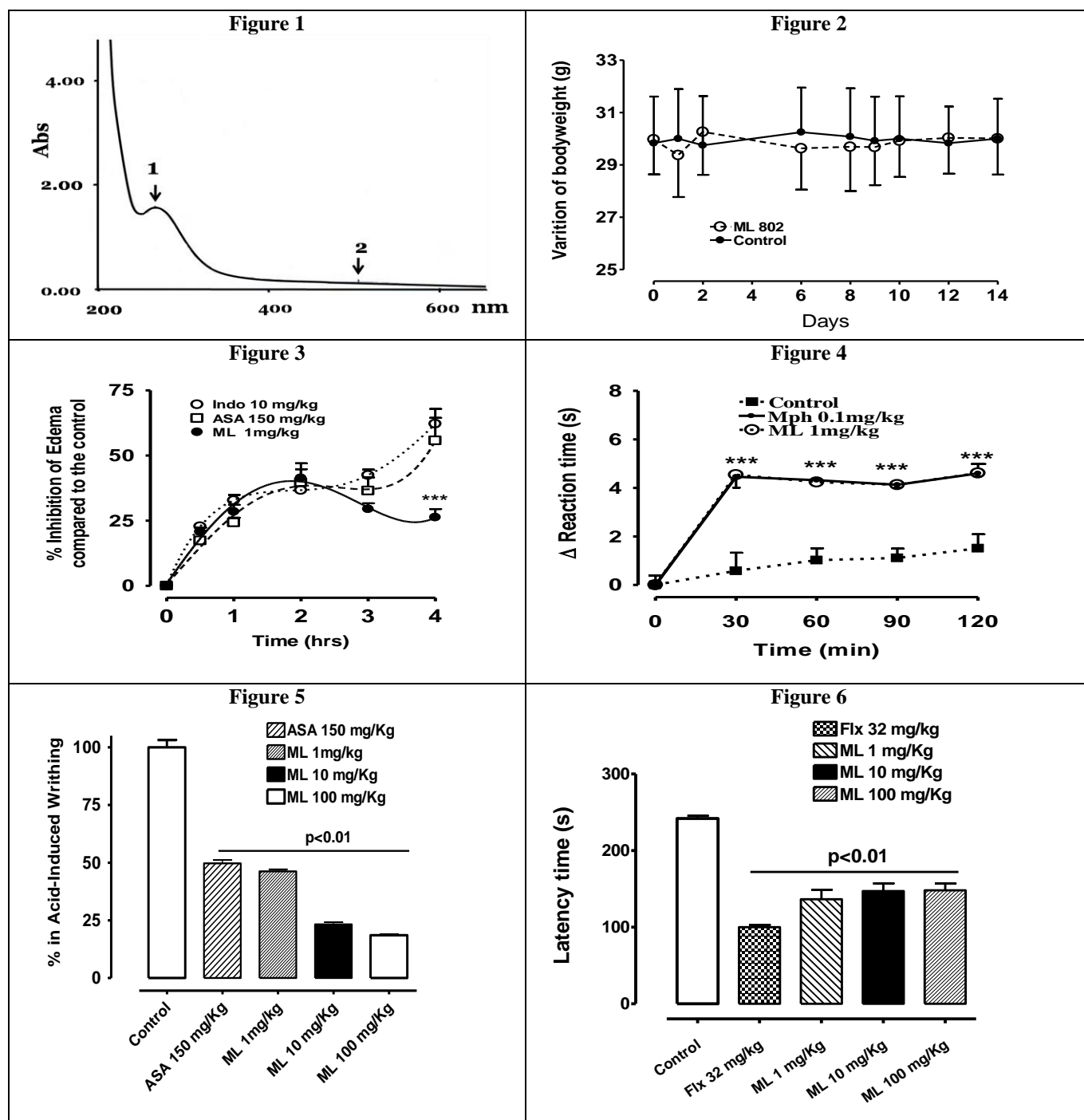
The central analgesic activity of LM was studied in tail withdrawal assay, as described by D'Amour and Smith (D'Amour & Smith, 1941). Radiant heat was applied to the base of the tail using a Tail-flick meter LE 7106 PANLAB (Harvard Bioscience, Spain). The latency time for removal of the tail from the stimulus was recorded. The intensity of the heat stimulus was set to elicit a tail flick within 10-12 sec. A cut-off time of 20 sec was used to prevent tissue damage. Mice weighing 20-30 g were randomly divided into 5 groups of 6 animals each. After recording the baseline latency (0 h), Groups II to IV were given ML at the rate of 1, 10 and 100 mg/kg. Group

I (control group) received comparable volume of vehicle (Isotonic NaCl 0.9%). Group V received the standard drug morphine hydrochloride (0.1mg/kg bodyweight, i.p.). The tail withdrawal latencies were measured at 0, 30, 60, 90 and 120 min, after the drug administration.

Statistical analysis

The whole data is expressed as a mean \pm

standard error of mean (S.E.M., n = number of experiments). The statistical analyses were obtained by the one way analysis of variance (ANOVA), followed by the Dunnett's test or Bonferroni post-tests, where necessary. $P < 0.05$ was considered significant. The concentration-response curves were analyzed by a non-linear regression (Graphpad program for Windows version 4.01. Graphpad, San Diego, CA, USA).



Legends to figures

Figure 1: Triterpene profile. The ML extract treated with boiled HCl. UV absorption spectra was recorded between 200-800 nm. The pick (1) was recorded at 265 nm.

Figure 2: Variation of bodyweight of mice after oral treatment of aqueous extract of ML (2g/kg) under regular observation for 14 days. Note that no variation was observed in treated animals compared to control.

Figure 3: Effect of ML on inflammation induced by λ -carrageenan in hind paws of mice. The result is represented in percentage of inhibition of edema compared to the control. The difference was evaluated using two-way ANOVA following Bonferroni post-tests, where $P < 0.05$ was considered significant.

Figure 4: Tail flick latencies in mice after administration of ML and morphine. The effect of LM is similar to that of morphine. The result is represented as difference (Δ) latency time between stimulation and reaction of mice. The difference was evaluated using a two-way ANOVA, following by Bonferroni post-tests, where *** $P < 0.001$ was considered significant.

Figure 5: Inhibition of Acetic acid-induced writhing by ML at different doses. Note that the effect of 1 mg/kg is similar to the effect obtained by 150 mg/kg of Acetylsalicylic acid (ASA). The difference was evaluated using One-way ANOVA following by Dunnett's multiple comparison tests. $P < 0.05$ was considered significant.

Figure 6: The effect of Fluoxetine and ML on depression induced by Forced swimming test. The time spent by a rat is reduced when animal received Fluoxetine and ML orally. The difference was evaluated using a One-way ANOVA, following Dunnett's multiple comparison tests. $P < 0.05$ was considered significant.

RESULTS

Phytochemical study for primarily qualitative analysis of active principles

Primarily qualitative analysis of active principles showed that ML did not contain saponosids, alkaloids, flavonoids, tannin, and anthracenosids. Only the presence of triterpene was detected. Examination of UV absorption spectra of extract ML after heating for 45 min in HCl (2N) showed the pick close to that of the known tetracyclic triterpene polycarpol (fig. 1), one of the triterpen identify in ML stem bark and some annonaceae family (Murphy *et al.*, 2008; Ngantchou *et al.*, 2009).

Acute toxicity

During the evaluation for acute toxicity of ML

neither 2.0 g/kg of orally administered resulted in death or any behavioural and/or physiological alterations, indicating that the extract has no/ or low toxicity. Histopathological analysis confirmed this result and the bodyweight did not differ from the control group (fig.2). But further studies will be performed to confirm the absence of chronic toxicity through haematological and biochemical parameters of blood.

Anti-Inflammatory Activity by Testing paws Oedema Induced by λ -carrageenan

The anti-inflammatory activity of ML was determined by the λ -carrageenan-induced paw edema test in the hind paws of mice. The results of orally administration of ML on λ -carrageenan-induced inflammation are represented in fig.3. The result showed that the effect of ML (1mg/kg) is similar to that of ASA (150mg/kg) and indomethacin (Indo 10 mg/kg) on reduction of inflammation (i.e. inhibition of paws edema) during 3 h. After 3h, this protective effect of ML decline ($P < 0.001$) compared to that of indomethacin and ASA, but it remains significant.

Evaluation of central analgesic activity by Tail flick method

The central analgesic activity of LM was studied in tail withdrawal assay, as described by D'Amour and Smith (D'Amour & Smith, 1941).

The results of orally administered ML (1mg/kg) on tail flick latencies in mice are summarized in fig. 4. The result shows that ML significantly increased the tail flick latency ($p < 0.001$) when compared to the control. The main finding is that the analgesic effect of 1 mg/kg of ML is similar to the positive control morphine (0.1mg/kg), suggesting that at 1mg/kg, ML can protect mice against pain similar to the morphine (01 mg/kg).

Evaluation of peripheral analgesic by acetic acid-induced writhing test

The oral administration of ML (1, 10 and 100 mg/kg) had a dose-dependent antinociceptive effect, and significantly decreased the number of writhing movements induced by the i.p. administration of the acetic acid, compared with the control group ($p < 0.01$). The percentages of inhibition were 53.77, 76.73 and 81.45 % for ML doses of 1, 10 and 100 mg/kg, respectively. Acetylsalicylic acid caused 50.31 % reduction in writhing movements (Fig.5).

Evaluation of anti-depressive effect of ML Using Forced swimming test

Analysis of variance (ANOVA) revealed significant differences ($p < 0.01$) between groups on the time spent by rats in treat arms compared to control

(fig.6). The latency time of Fluoxetine (Flx) is 100 ± 9.65 sec versus control 242 ± 11.8 sec. Oral administration of ML (1 mg/kg) was 136.50 ± 39.04 and remain stable when the concentration of ML increase.

DISCUSSION

This study was designed to verify the antinociceptive, antidepressant, anti-inflammatory effect and acute toxicity of extract of *Meiocarpidium lepidotum* (ML) in rats and mice. The result showed the analgesic, anti-inflammatory and antidepressant effect of ML. This study is the first conducted by a biological screening of aqueous extract of ML. Although ML is widely use traditionally for making various devices (Leboeuf *et al.*, 1977b), there is no significant literature on the traditional use of this plant as a phytomedicine.

The first phytochemical screening of this plant was done 1977 by Leboeuf *et al.* (Leboeuf *et al.*, 1977a). They were the firsts to demonstrate that phytochemical screening of the stem bark, a root for herbarium of the Congo (Herbier Paul Sita 3337, and Herbier P.S 3628), did not contain any flavonoids, saponosids, tannins, and quinones. They, nevertheless, found triterpenic and derivatives compounds. They also found steroids (Liebermann reagent), and alkaloids (Meyer and Dragendorff reagent). Furthermore, they found that triterpene and a derivative were polycarpol or hydroxyl-15 α agnosterol. Those two compounds (alkaloid and polycarpol) were also found in other African Annonaceae: *Polyalthia oliveri* Engl (Quevauviller & Hamonniere, 1977), and *Pachypodanthium confine* Engl & Diels (Bevalot *et al.*, 1977; Quevauviller & Hamonniere, 1977), *Isolona campanulata* Engl. & Diels (Hocquemiller *et al.*, 1984). Our study confirmed the presence of triterpene, but no flavonoids, saponosids and tannins as well. But our extract did not contain alkaloids because we used aqueous extract with only distilled water. The extraction of alkaloids of ML was done with only organic solvents.

During the evaluation for acute toxicity of ML neither 2.0 g/kg of orally administered resulted in death or any behavioural and/or physiological alterations, indicating that the extract has no/ or low toxicity. But further studies will be performed to confirm the absence of chronic toxicity through haematological and biochemical parameters of blood.

Anti-Inflammatory Activity by Testing paws Oedema Induced by λ -carregeenan. Carrageenan-induced paw edema, an *in vivo* model of inflammation, has also been characterized as a biphasic event (Vinegar *et al.*, 1969). Histamine, bradykinin, and 5-hydroxytryptamine (5-HT) are released in the first phase of edema (0-1 h). In the second phase (1-6 h), TNF- α , IL-1 β , cyclooxygenase (COX-2), and prostaglandin (PGs) are produced more actively (Chang *et al.*, 2012). It is well known that the expression of COX-2 is maximal at the late phase of λ -

carrageenan induced paw edema, which could subsequently increase prostaglandin levels in inflammatory reactions (Seibert *et al.*, 1994). In this study, ML (1mg/kg), ASA (150 mg/kg) and indomethacin (10 mg/kg) showed significant anti-inflammatory effect on λ -carrageenan induced mouse paw edema from the 3 h and their effect are similar. In contrast after 4h, the effect of ML decline compared to that of indomethacin and ASA. According to the hypothesis that up to 1h of inflammation induced by λ -carrageenan, TNF- α , IL-1 β , COX and PGs is produced more actively. In this phase, the activity of ML decline. We can postulate that the activity of ML is reduced in the presence of TNF- α , IL-1 β , COX and PGs, and was higher in the presence Histamine, bradykinin, and 5-HT. In contrast, NSAIDs, such as indomethacin and acetylsalicylic acid, seem to suppress only the second phase (Bogdan, 2001).

In this study, to distinguish between the central and peripheral antinociceptive action of ML, two different tests was performed. The acetic acid-induced writhing test was used to evaluate the antinociceptive activity of ML. The intraperitoneal administering of agents that irritate serous membranes provokes a stereotyped behaviour in mice, which is characterized by abdominal contractions, movements of the body and twisting of the dorso-abdominal muscles. It is the typical pain generated indirectly via endogenous mediators, a suck as bradykinin, serotonin and capsaicin, which stimulate peripheral nociceptive neurons. The releases of arachidonic acid metabolites via COX and PGs biosynthesis are involved. ML reduced acetic acid-induced writhing in mice. This result suggests that, the extract from ML may act by inhibiting PGs synthesis because the nociceptive mechanism of abdominal writhing induced by acetic acid involves the release of arachidonic acid metabolites via COX. But we cannot determine with this test if the activity of ML is of central or peripheral origin. Evaluation of central analgesic activity was done by Tail flick method. The increase of duration of the time of reaction is a characteristic of the drugs that act centrally. It indicates a possible interaction with opioid receptors. When ML, at 1mg/kg, was administrated to mice, we obtained a similar result when we compared it with the effect of 0.1mg/kg of morphine. This result suggests that ML may act as opioid analgesics.

Evaluation of anti-depressive effect of ML was realized in the forced swimming test. Groups taking Fluoxetine orally, and ML, decreased the immobility (latency) time. This potentiation manifested as a reduction of the immobility time in the forced swimming test with no influence on locomotor activity (Poleszak *et al.*, 2011). During the test, the antidepressant compounds (Fluoxetine) and ML did not alter or reduce the locomotor activity. In addition, rats given drug treatment displayed significantly less immobility and stops than the control

rats. The immobilization represents a state of desperation in the rodents, which is a symptom of depression. Porsolt *et al.* (Porsolt *et al.*, 1978) found that such immobility is reflective of a low-mood state in rats, which is sensitive to antidepressant treatment. However, rats given antidepressant drug treatment and ML displayed significantly less immobility and stops than the control rats.

Furthermore, behavioural registration of the time spent swimming in the tank or climbing (attempted vertical movement) allows the detection of the selective serotonin reuptake inhibitors, and the discrimination between drugs affecting primarily the serotonergic or noradrenergic neurotransmitter systems (Page *et al.*, 1999; Lopez-Rubalcava & Lucki, 2000; Reneric *et al.*, 2001). While drugs stimulate the serotonergic system, such as selective serotonin reuptake inhibitors, and stimulate the active swimming in the water tank, they, primarily, block the noradrenalin uptake and, preferentially, increase the climbing behaviour. The selective serotonin reuptake inhibitor Fluoxetine boosted swimming behaviour. The effect of ML is similar, but less powerful than that of the Fluoxetine.

The monoamine theory of depression has

predominated with regards to the aetiology of the illness itself, as well as the rationale behind most treatments is available in clinics. Currently, some of the most widely prescribed antidepressant drugs are those with high degrees of selectivity for the 5-HT transporter, the selective serotonin reuptake inhibitors (eg, fluoxetine) and, to a lesser extent, those with a high degree of selectivity for the noradrenalin transporter, the selective noradrenalin reuptake inhibitors (Haenisch & Bonisch, 2011). The mechanism of action of ML was unknown but further studies will be performed to confirm this mechanism.

CONCLUSION

In conclusion, *Meiocarpidium lepidotum* (ML) is effective as an analgesic agent in various typical pains. The antinociceptive effect of ML is mediated via inhibition of peripheral mediators and central inhibitory mechanisms. In addition, ML appears to exhibit anti-inflammatory and antidepressant properties. Our results support that ML has therapeutic potential for the treatment of painful disorders and depression. Further studies, currently in progress, will enable us to understand the precise mechanisms of action of ML.

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