Original Research Article

The mixture aqueous extracts from *Oxalis corniculata L.* and *Acmella caulirhiza* Delile accelerates bone healing in fractured rats.

Abstract

Aim: To investigate the effect of the mixture aqueous extracts from *Oxalis corniculata* and *Acmella caulirhiza* on bone formation.

Methodology: Wistar female rats were fractured using a drill machine by inserting a drill bit in the femur diaphysis. Fractured animals were subdivided into five groups. One group receiving palm oil, four groups receiving plant extract at the doses of 150, 300, 600 mg/kg or diclofenac at the dose of 357 mg/kg. Substances were given via percutaneous and oral routes for two weeks. At the end of the experimental period, bones were collected and homogenized for the evaluation of the following biochemical parameters: alkaline phosphatase (ALP); reduced glutathione (GSH), nitrites, superoxide dismutase (SOD), interleukin-1β and interleukin-6 Moreover, histomorphometry of the bone at the fracture site was realized.

Results: Percutaneous treatment with the plant extracts resulted in a significant increase of ALP ($p<0.05$) activity in fractured rats as compared to the control. The mixture extracts also alleviated the fracture-induced oxidative stress by increasing the concentrations of GSH and nitrites; SOD and catalase activities. The plant extracts improved deregulated cytokines observed in fractured bone. Histopathological examination, showed a more pack-like structured with a significant decrease in trabeculae number, trabecular interspace with the increase in the bone thickness.

Conclusion: The mixture aqueous extracts from *Oxalis corniculata* and *Acmella caulirhiza* possesses bone healing effect due to its ability to alleviate oxidative stress, regulate pro-inflammatory cytokines and improve the structure of the new bone formed.

Key word: Fracture, *Oxalis corniculata*, *Acmella caulirhiza,* Stress, Cytokines, histomorphometry

1.Introduction

Bone tissue is a specialized and dynamic tissue in perpetual renewal that undergoes a constant process of remodeling to accommodate changing mechanical stresses [1]. The broken bone is allowed to regenerate naturally on its own due to a complex process such as cell and tissue proliferation and differentiation, factors including growth factors, inflammatory cytokines, antioxidants and nutrients. Bone injury results a secretion of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukins (IL-1, IL-6, IL-11, IL-23), bone morphogenetic proteins (BMPs). These molecules act together to stimulate biological process at the fracture site, recruiting macrophages, monocytes, and lymphocytes [2, 3]. Till date, fracture management involves several stages: the stabilization of the fracture site, by using inflatable plastic braces; tools clinical and / or radiographic examinations, allowing the orientation of the choice of treatment which can be orthopedic and / or surgical; drug treatments consist of using osteoinductive (bisphosphonates, synthetic bone morphogenetics mroteins) and anti-inflammatory substances[4, 5]. The administration of calcium and vitamin D is also considered, with the patient being kept on drugs [6]. In clinical application, these treatments possess a limitation. Medicinal plant remains an important source of a primary healthcare mode for around 85% of the world's population [7]. The use of these plants is also the first material for some pharmaceutical industries. Thus, about 80% of all synthetic drugs derived from medicinal plant

[8, 9]. Considering the powerful pharmacological effect of many plant extracts already shown; Several research teams continue to explore the ethnopharmacological data provide by the traditional healer to scientifically demonstrate the veracity of theses biological effects by using experimental models. Our research team, after an ethnopharmacological survey has previously shown a therapeutic effect of a Cameroonian plant extract on bone fracture [10]. Facilitating bone healing process is benefic for the patient to rapid recover and to reduce the cost of and time treatment. In west Cameroon, traditional healers used plant extract alone or in combination to manage fracture. Thus, the mixture of powders of *Acmella caulirhiza* and *Oxalis corniculata* (Oxalidaceae) in palm oil is used, in this region for the treatment of bone fractures.

Oxalis corniculata (Oxalidaceae) is empirically used for amydalites. Further studies revealed its anti-inflammatory, anti-diabetic, anti-ulcerogenic, antibacterial and antioxidant properties [11-13]. Apart from diuretic property, anti-fungal, hepatoprotective, anti-diabetic and antioxydant effect of *Acmella caulirhiza* (Asteraceae) [14], the results of previous research have shown that the ethanolic extract of the plant accelerates fracture repair [10]. No scientific data has reported the effect of *Oxalis corniculata* alone or in combination with *Acmella caulirhiza* on fracture healing. The present study investigated the effect of extract mixture from *Acmella caulirhiza* and *Oxalis corniculata* in palm oil on fracture healing.

2.Materials and methods

2.1.Plants material

Whole plants of *Oxalis corniculata* and *Acmella caulirhiza* were harvested at Bafoussam III in the West region of Cameroon in March 2019. Plants were authenticated at the Cameroon National Herbarium in comparison with the specimen voucher N° 8680 / SRF/cam and N° 3307617NC respectively for *Oxalis corniculata* and *Acmella caulirhiza*. The whole fresh plant

were cleaned, cut into pieces and dried under a shade at room temperature. The decoction was carried out separately by boiling 100 g of the powder in 1.5 L of tap water for 10 min following the traditional healer instructions. The filtrate obtained was dried at 45°C in drying-cupboard to yield 20% and 24.14% respectively of *Oxalis corniculata* and *Acmella caulirhiza* extract; kept at room temperature until use. The dose of the traditional healer was determined and corresponding to 300 mg/kg which was surrounding by the lower (150 mg/kg) and the higher (300 mg/kg) dose. After preparing different tested doses, a third of each dose was administrated by percutaneous route while the two third were given orally as recommended by the practionner.

2.2.Animals

Male rats weighing 250 ± 20g were used in the present study. They were obtained from the animal house of Faculty of Science at the University of Yaoundé I (Cameroon). Animals were submitted to the standard diet established in this laboratory and they received water ad libitum. The procedures followed the principles of laboratory animal use and care of the "European community guidelines (EEC Directive 2010/63/EEC) and were approved by the "Animal Ethical committee" of the Faculty of Science, University of Yaoundé I.

2.3.Femoral drill hole injury

All surgical equipment used to induce the fracture were overnight soacked in alcohol to avoid an eventual infection. A drill hole injury was created as described by Ngueguim et al.[15, 16]. Briefly, the front skin of the mid femur in rats under anaesthesia was incised. After splitting the muscle, periosteum was stripped to expose the femoral bone surface. A drill-hole injury was created using a drill machine (Electrex) by inserting a drill bit with a diameter of 1.2 mm in the anterior portion of the diaphysis of one femur [17]. Fractured animals were subdivided into five

groups of seven rats treated as follow: one group treated with palm oil (10 mL/kg), another group was treated with sodium diclofenac (357 mg/kg) and three groups were treated with the mixture of the plant extracts at the doses of 150, 300 or 600 mg/kg. In the plant mixture, *Acmella caulirhiza* and *Oxalis corniculata* represented respectively 52.5% and 47.5% as recommended by the traditional healer. Two groups of unfractured were treated respectively with palm oil (10 mL/kg) or the plant extract at the dose of 600mg/kg. Different substances were daily administered by percutaneous (1/3 of the dose) and oral (2/3 of the same dose) routes for two weeks. At the end of experimental period, all animal were sacrificed under anaesthesia using ketamine (30 mg/kg) and valium (10 mg/kg) via intraperitoneal route. One part of the fractured femur (per group) were collected and refrigerated at −20 °C for bone homogenates using 0.1 M PBS. The other part of fractured femur was used for histopathological examination and static histomorphometry at the fracture site.

2.4.Assessment of the mixture aqueous extracts from *Acmella caulirhiza* and *Oxalis corniculata* on some bone parameters of oxidative stress

Bones were carefully removed and the femur has been gently stripped of muscle tissue; the distal portion containing fracture site was weighted (0.2 g). The organ was ground on a grinding stone, which had beforehand been covered entirely with a hard, transparent, plastic paper. A volume of 3 mL of PBS (phosphate buffer saline) was added to the paste. The bones homogenate were then centrifuged at 3000 rpm at 4 ° C for 30 min. The supernatant obtained was used for for biochemical analyses such as alkaline phosphatase, anti-oxidative parameters including: superoxide dismutase (SOD), catalase, reduced glutathione (GSH) and nitrites using commercial kit (Biolab).

2.5.Bone inflammatory parameter analysis.

Bone homogenate was centrifuged, and supernatant was used for some inflammatory parameters measurement such as IL-1β and Il-6 using Quantikine Elisa kits (Germany). Briefly, fifty microliter of assay diluent (RD1-54 and RD1-21 respectively for IL-6 and IL-1β) were added into ELISA pre-coated (antibody Il-6 or IL-1β specific for rat) plate. Then, fifty microliter of the standard or the sample were added to the correspondent well. The mixture was gently homogenized for one minute and the microplates were incubated at 37°C during two hours. Wells were washed five times (with a washer buffer provided by the manufacturer). Moreover, 100 µL of rat specific antibodies anti-Il-6 or anti-IL-1β conjugate to horseradish peroxidase were respectively introduced in each well and the mixture was incubated for two hours; the preparation was then washed and incubated with streptavidin for thirty minutes. The enzyme reaction was stopped using sulfuric acid solution. The optical density of each well was measured using a microplate reader .The concentration of each cytokines was determined from the corresponding calibration curve.

2.6.Histopathological analysis

Histological analysis at the site of the fracture was assessed by Haematoxilin-Eosin staining method. The bones were conserved in 10% formalin and demineralized in 10% HCL solution for 5 days. Isolated femur samples containing the drill hole injury were embedded in paraffin and a thickness of 10 µm sections were made using a microtome (Reichert-Jung 2030). Photographs of the sections were taken using a digital camera for microscope (DCM 35:350 K Pixels, USB 2.0) aided with appropriate filters.

2.7.Assessment of static histomorphometry

The thickness and the number of trabeculae were measured using ImageJ software [18] (ImageJ, version 1.49). The thickness of the trabeculae was carried out by performing twenty measurements per field (four) in each slide. Then the averages were considered.

2.8.Statistical analysis

Results are expressed as mean ± standard error mean. Statistical significance was determined by one way analysis of variance followed by the Tukey post-test using Graph pad Prism version 8.0.1 (GraphPad Software, San Diego, California, USA). Differences were considered significant at $p < 0.05$.

3.Results

3.1Effects of the mixture of *Oxalis corniculata* and *Acmella caulirhiza whole plant aqueous extracts on bone alkaline phosphatase*

Figure 1 illustrates the effect of the mixture of *Oxalis corniculata* and *Acmella caulirhiza* whole plant aqueous extract on bone alkaline phosphatase. Fractured rats showed a significant reduction of ALP activity ($p< 0.05$) compared to unfractured control rats. The percutaneous administration of the plant extract at the doses of 150 and 300 mg/kg for two weeks provoked a significant increase ($p< 0.05$) level of ALP activity respectively by 28.62 % and 28.06 % compared to fractured control. No significant change was observed at the dose of 600 mg/kg in fractured and unfractured rats. The administration of diclofenac at the dose of 357 mg/kg to fractured rats induced a significant increase ($p< 0.01$) of ALP activity.

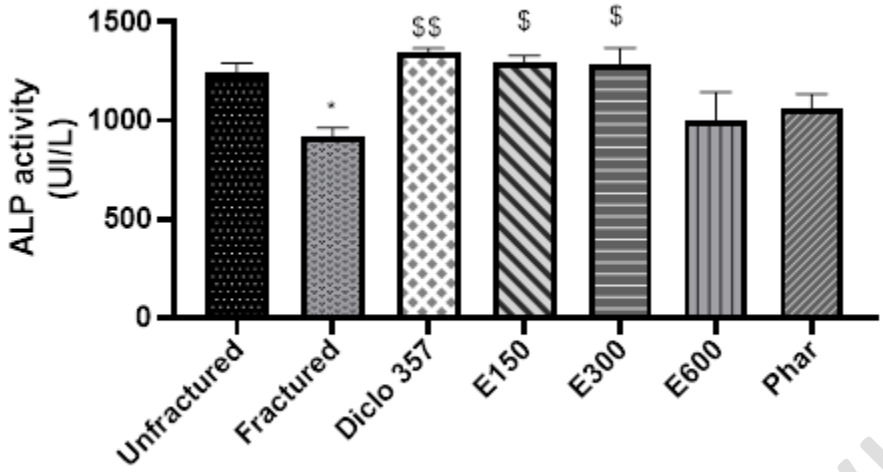

Figure 1: Effects of the mixture of *Oxalis corniculata* and *Acmella caulirhiza* whole plant aqueous extracts on bone alkaline phosphatase at the fracture site.

Each bar represents mean ± SEM (n = 7). * $p < 0.05$: significant different compared to unfractured rats ; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$: significant different compared to fractured rat. E150, E300 and E600: fractured rats treated with the mixture plant extract at the dose of 150, 300 600 mg/kg; Diclo357: fractured treated with diclofenac at the dose of 357 mg/kg; Phar= unfractured rats receiving the extract at the dose of 600 mg/kg.

3.2.Effects of the mixture of *Oxalis corniculata* and *Acmella caulirhiza* whole plant aqueous extracts on some non-enzymatic parameters of oxidative stress

Bone injury provoked a significant decrease in reduced glutathione level by 51.31% ($p< 0.001$) in comparison to unfractured control rats (Fig 2A). The treatment with the plant extracts mixture at the doses of 150 and 300 mg/kg induced a significant increase of reduced glutathione level by 38.28 % ($p< 0.01$) and 30.32 % ($p< 0.05$). It was noticed that the level of reduced glutathione was significantly ($p< 0.001$) low as compared to unfractured rat. In comparison to unfractured

rats, the concentration of bone nitrites at the site of fracture was reduced by 39.68 % ($p< 0.05$) (Fig. 2B). The administration of the extract at the dose of 150 mg/kg significantly increased nitrites concentration by 42.42 % ($p<0.05$). Percutaneous administration of the extract at the dose of 600 mkg/kg failed to increase both parameters in fractured rats.

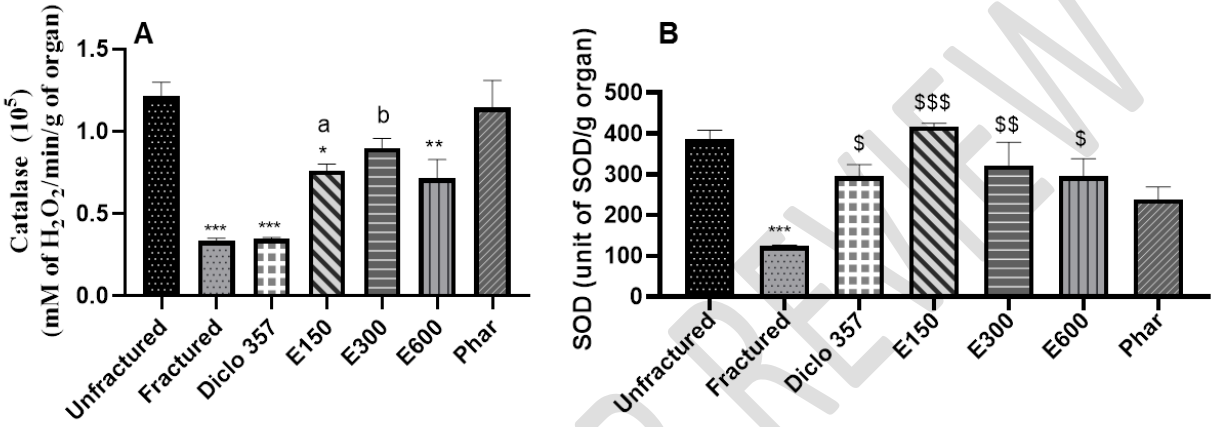

Figure 2: Effects of the mixture of *Oxalis corniculata* and *Acmella caulirhiza* whole plant aqueous extracts on reduced glutathione (A) and nitrites (B) at the fracture site

Each bar represents mean ± SEM (n = 7). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$: significant different compared to unfractured rats ; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$: significant different compared to fractured rats. E150, E300 and E600: fractured rats treated with the mixture plant extracts at the dose of 150, 300, 600 mg/kg. Diclo357: fractured rats treated with diclofenac at the dose of 357 mg/kg; Phar= unfractured rats receiving the extract at the dose of 600 mg/kg.

3.3. Effects of the mixture of *Oxalis corniculata* and *Acmella caulirhiza* whole plant aqueous extracts on some enzymatic antioxidant parameters

Enzymatic antioxidant parameters such as SOD and catalase were measured at femoral fracture site two weeks post-fracture (Figure 3). Fracture induction showed a significant decrease in

catalase activity by 72.70 % ($p < 0.05$) when compared to unfractured rats. However daily percutaneous administration of the plant extracts mixture exhibited a significant increase in catalase activity by 56.18 % ($p < 0.05$) and 64.14 % ($p < 0.01$) at the respective doses of 150 mg/kg and 300 mg/kg. The SOD activity was significant decreased in fractured rats (Fig 3B). The plant extract treated groups showed a significant raise in the SOD activity. The increase was by 70.26 % ($p < 0.001$), 61.49 % ($p < 0.01$) and 47.90 % ($p < 0.05$) at the respective doses of 150, 300 mg/kg and 600 mg/kg. Diclofenac used as anti-inflammatory drug failed to correct catalase and SOD activities in fractured rats.

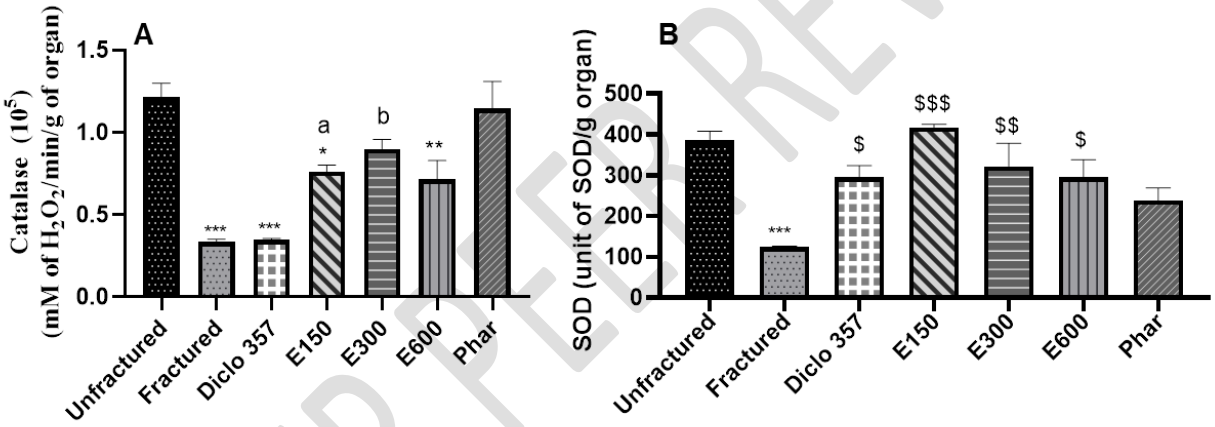

Figure 3: Effects of the mixture from *Oxalis corniculata* and *Acmella caulirhiza* whole plant aqueous extracts on Catalase (A) and SOD (B) activities at the fracture siteEach bar represents mean ± SEM (n = 7). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$: significant different compared to unfractured rats ; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.01$:significant different compared to fractured rats. E150, E300 and E600: fractured rats treated with the mixture of plant extracts at the doses of 150, 300, 600 mg/kg Diclo357: fractured rats treated with diclofenac at the dose of 357 mg/kg; Phar= unfractured rats receiving the extract at the dose of 600mg/kg.

3.4. Effects of the mixture of *Oxalis corniculata* and *Acmella caulirhiza* whole plant aqueous extracts on some pro-inflammatory cytokines parameters

Two weeks following the fracture, control fractured group showed a significant increase ($p < 0.01$) rate of IL-1β by 85.92% (Fig 4A) and IL-6 by 87.41% (Fig 4B). The administration of the mixture of extracts for two weeks showed a significant reduction of IL-1β and IL-6 by 82.34% and 89.29% at the extract dose of 150 mg/kg and, 75.41% and 84.41% at the dose of 300 mg/kg. The administration of diclofenac at the dose of 357mg/kg in the same experimental conditions significantly reduced the rate of IL-1β and IL-6 by 62.40% and 80.51% as compared to the fractured rats.

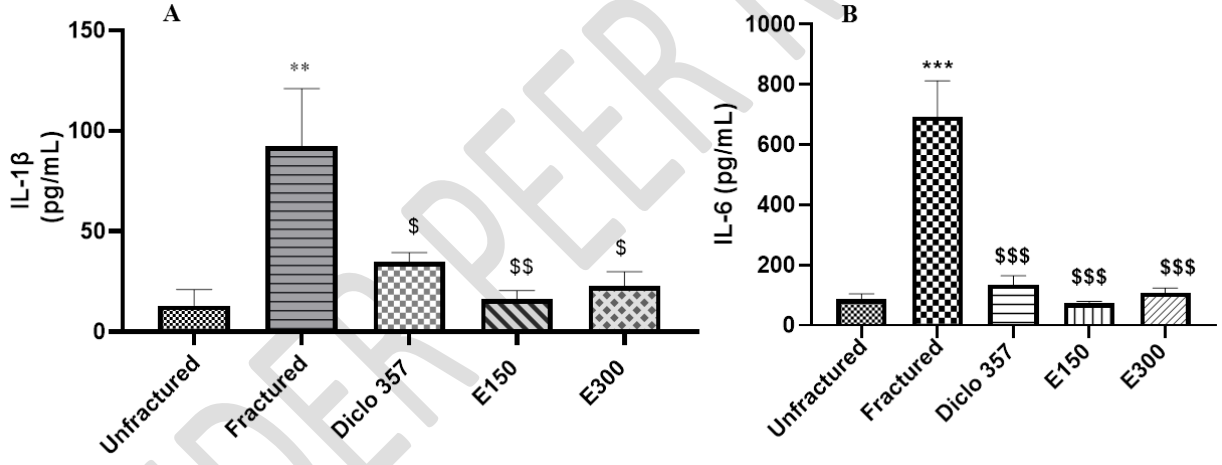

Figure 4: Effects of the mixture of *Oxalis corniculata* and *Acmella caulirhiza* whole plant aqueous extracts on IL-1β (A) and IL-6 (B) activities at the fracture site.

Each bar represents mean ± SEM (n = 7). ** $p < 0.01$; *** $p < 0.001$: significant different compared to unfractured rats ; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.01$: significant different compared to fractured rats. E150, E300 and E600: fractured rats treated with the mixture of plant extracts at

the doses of 150, 300 and 600 mg/kg Diclo 357: fractured treated with diclofenac at the dose of 357 mg/kg; IL-1β = interleukin 1 beta, IL-6 = interleukin 6.

3.5.Effects of the plant extracts mixture on the number, thickness of trabecular bone and the intertrabecular space

Figure 5 showed the number, the thickness and the intertrabecular space of new bone formed at the fracture site of treated groups (Figure 6 A, B & C). The trabeculae number in fractured control increased while the thickness decreased. Fractured animals treated with the extracts mixture exhibited a significant ($p< 0.001$) decrease in trabecular number by 63.98 %, 55.91 % and 51.30 % respectively at the doses of 150 mg/kg, 300 mg/kg and 600 mg/kg; whereas the thickness of trabecular bone significantly increased ($p< 0.001$) by 41.94 (150 mg/kg), 40.89 % (300 mg/kg) and 42.94 %(600 mg/kg) in comparison to fractured untreated control. Moreover, Intertrabecular space elevated in fractured group was significantly reduced in treated groups ($p< 0.001$) with a pronounced effect at the dose of 150mg/kg.

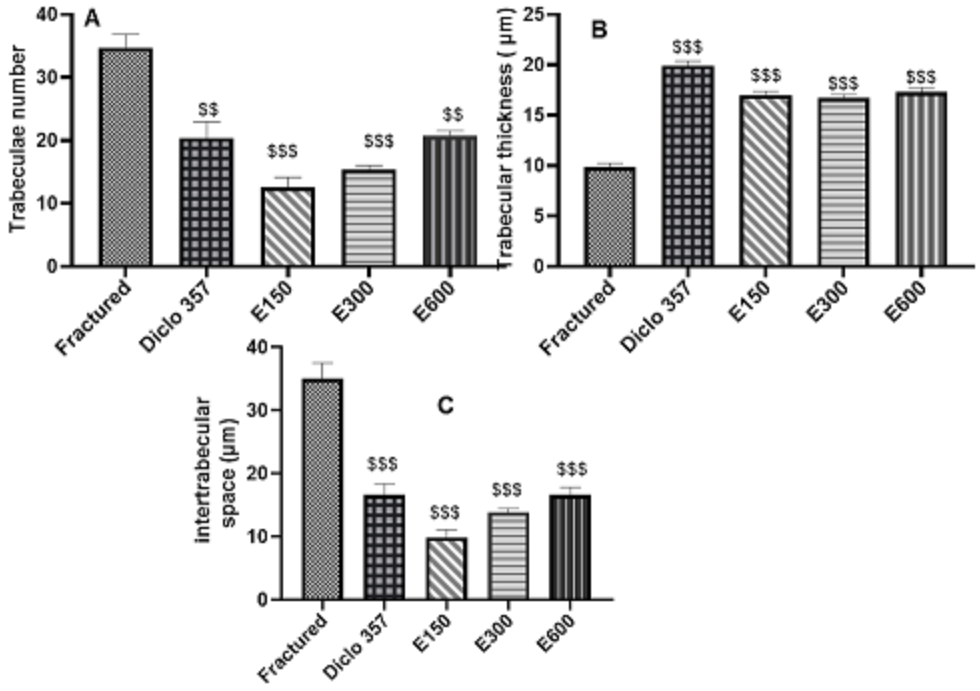

Figure 5: Effects of the mixture of *Oxalis corniculata* and *Acmella caulirhiza* whole plant aqueous extracts on the number (A), thickness of trabecular bone (B) and the intertrabecular space (C) at the fracture site.

Each bar represents mean ± SEM (n = 7). $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.01$: significant different compared to fractured rats. E150, E300 and E600: fractured rats treated with the mixture of plant extracts at the doses of 150, 300 and 600 mg/kg, Diclo 357: fractured treated with diclofenac at the dose of 357 mg/kg.

3.6.Effects of the plant extracts mixture on histological examination

Fractured rats presented a callus characterized by a very loose bone structure with a wider trabecular space as compared to normal bone architecture (Fig 6A). In the treated groups at the

doses of 150 and 300 mg/kg (Fig 6B & Fig6C) trabecular bone were fused with a reduction of trabecular space. The effect was marked at the dose of 150 mg/kg. Fractured animal receiving diclofenac showed a bone structure closed to that of unfractured animals.

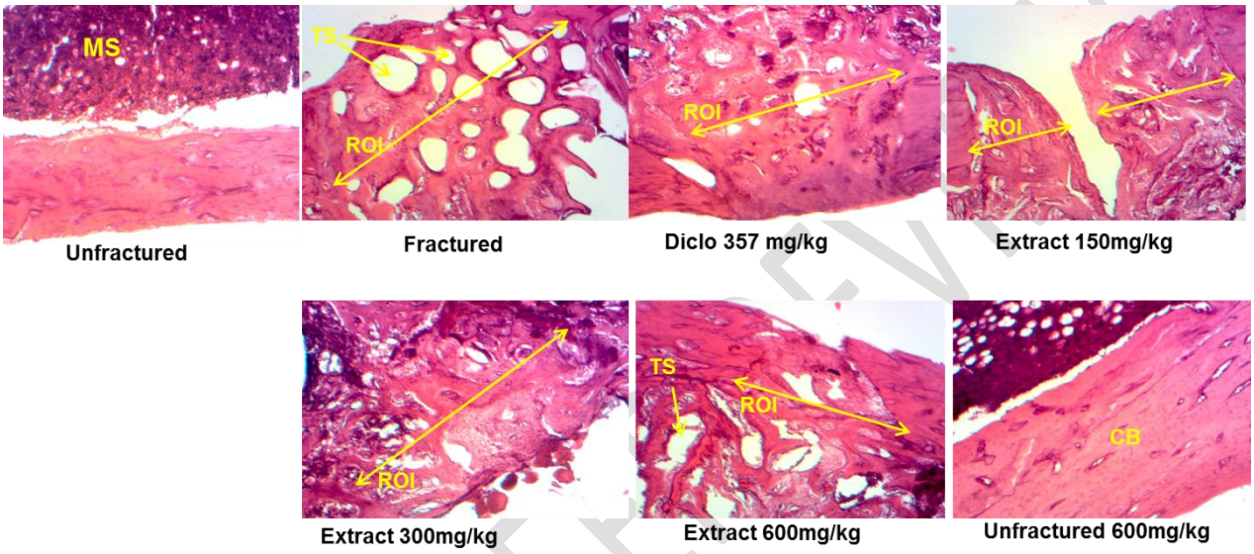

Figure 6: Effects of the plant extracts mixture on bone callus formation (H&E stain x25).

E150, E300 and E600: fractured rats treated with the mixture plant extract at the dose of 150, 300, 600 mg/kg Diclo357: fractured treated with diclofenac at the dose of 357 mg/kg; Unfractured 600mgkg: rat without fracture receiving the extract at the dose of 600 mgkg. MS: medullar space, TS: trabecular space

4.Discussion

Oxalis corniculata and *Acmella caulirhiza* are both herbaceous plants used each alone for various ailments. The mixture extracts of these two plants is used in Cameroonian west region

for fracture healing, and no biological activity was studied up to now yet. Therefore, the present study aimed to evaluate the effect of *Oxalis corniculata and Acmella caulirhiza* aqueous extracts mixture for bone healing effect. The result showed a decrease of alkaline phosphatase activity in fractured rat. This enzyme is an indicator of bone formation, mainly produced by pre-osteoblast and osteoblast confirming the proliferation and the differentiation of mesenchymal stem cells [19]. The mixture extracts of plants increased the ALP activity suggesting the probable benefic effect of these extracts mixture on mesenchymal stem cells and consequently on bone formation. Fracture result to a blood vessels rupture, causing ischemia and hypoxia on surrounding environment [20, 21]. As result to these processes, is the formation of new vessels which causing oxygen overload, thus oxidative stress at the fracture site. Several studies demonstrated that oxidative stress exist during fracture healing process mainly in the stage of callus formation [21, 22]. Oxidative stress can be evaluated by measuring, the end product of lipid peroxidation malondialdehyde (MDA), with non-enzymatic markers such as 8-hydroxy-2-deoxyguanosine (8-OHdG), oxidative DNA damage indicator, protein oxidation SOD, GPx, GST, GR and antioxidant enzymes such as ascorbic acid, reduced glutathione, ubiquinone, and cysteine[22]. In the present study, the fracture induced a significant decrease in GSH and nitrite levels, catalase and SOD activities. The decrease in GSH level may be due to the overproduction of reactive oxygen species (ROS) and/or an increase in the harmful effects of hydrogen peroxide following the inhibition of glutathione peroxidase activity [23].The decrease in nitrites level could be due to the cell damage at the fracture site and/or the inhibition of iNOS by inflammatory activity at bone fracture. While the decrease in catalase and SOD activities is thought to be due to an overproduction of superoxide anion and hydrogen peroxide after reperfusion [24, 25]. These results provided the justification of the existence of stress after a bone hole injury contributing to

slowing bone consolidation. It is well know that inflammation response is one stage of fracture healing and during this stage, ROS produced can in turn cause further damage or can exacerbate the stress-inflammation cycle [21]. When fracture occurs, there is a cascade of events involving the production of cytokines. In fact, Studies have shown that during fracture healing process, inflammatory cells proliferate and migrate to the fracture site, initiate a series of healing reactions [3, 26]. Among these cells are interleukin-1 and interleukin-6 .These cells are key pro-inflammatory cytokines whose role is to promote the occurrence of inflammation, and cause infiltration of inflammatory cells as well as induce the proliferation and differentiation of stem cells[3]. Osteoblasts and chondroblasts release cytokines within 3-7days following the fracture [26, 27]. However in this study, the level of IL-1β and IL-6 in fractured rats remained higher than those of unfractured rats suggesting a lower and progressive natural healing process. The significant reduction of IL-1β and IL-6 very closed to normal value supporting the view that the mixture aqueous extracts from *Oxalis corniculata* and *Acmella caulirhiza* accelerates bone healing process. These different effects of the extract could be attributed to the presence of antioxidant and anti-inflammatory compounds in the mixture. In fact, flavonoïds, sterols and terpenoides within *Acmella caulirhiza* [28]; phenols and tanins within *Oxalis corniculata* [29] are group of compounds known for their ability to scavenge ROS. These compounds have probably reacted synergically to accelerate bone healing. Several methods are used for assessing skeletal repair including histomorphometry [30, 31]. Thus, in this study, the pharmacological effects observed is reinforced by the reduced number of formed trabeculae, their thickness and the trabecular space. This result is strengthening by a fused bone callus observed in histologic microphotograph. In addition, the reduction of the trabecular space is the consequence of the increase of thickness of trabeculae attesting a more pack-like structure of new bone formed. The

aqueous extracts mixture of *Oxalis corniculata* and *Acmella caulirhiza* possesses a double potential: anti-oxidant and anti-inflammatory than the reference drug which acts only as anti-inflammatory. These two pharmacological effects of the plant extract mixture could explain the efficacy of the extract.

In conclusion, bone injury was characterized by a decrease in ALP activity, an increase in oxidative stress accompanied by an increase in cytokine such as IL-1β and IL-6 with a loose bone structure. The administration of the aqueous extracts mixture of *Oxalis corniculata* and *Acmella caulirhiza* significantly increased ALP activity, improved antioxidant defense and bone architecture at fracture site. This bone healing effect observed in the present work was achieved by the antioxidant and antiinflammatory activities of the extract justifying the use of this extract mixture in the management of the fracture.

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