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The Whole Plant Aqueous Extract of *Cleome Rutidosperma* (Cleomaceae) Improves bone Formation in Ovariectomy-Induced Fragility Fracture in Rats

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Abstract: Background:Osteoporosis is one of the major factors ofbonemicroarchitecture alteration leading to an increase risk of fractures. The present studyassesses the effect of the aqueous extract of *Cleome rutidosperma*(*C. rutidosperma*)on osteoporotic fractures. ***Methodology***: Female rats were ovariectomizedand 84 daysafter the development of osteoporosis, a hole was created inthe femur. The ovariectomized-fracturedgroup received distilled water (10 mL/kg); extract (75 mg/kg, 150 mg/kg or 300 mg/kg)or estradiolvalerate(1mg/kg) orally for twenty-eight daysand thenthe animals were sacrificed. Radiological(femur)and histological (femur fracture site and femoral head)analyses were performed.Biochemical parameters (calcium, phosphorus, and ALP activity) some oxidative stress parameters(malondialdehyde, reduced glutathione, catalase, and nitrite); TGF-β, TNF-α,and osteocalcin levels were evaluated.Phytochemical analysis was performed using LC/ESI-MS analysis.

***Results*:**The ovariectomized-fracturedratsshowed a lipid profile altered($p<0.001$), a reduction in bone mineral density; deterioration of bone formation markers accompanied by femoral head bone resorption with loose bone regeneration at the fracture site. Theadministrationofthe plant extract improved lipid profileandinduced a significant increase in bone density with an improvement in ALP activity. In addition, the extract reduced oxidative stress by increasing GSH, nitrite concentrations, and catalase activity,whileMDA levels decreased.The extract increased TGF-β ($p<0.01$-$p<0.001$), and osteocalcin ($p<0.01$-$p<0.001$) levels while TNFαdecreased ($p<0.001$) with a marked effect at the dose of 75mg/kg. At this dose, the extract showed the disappearance of the resorptive area in the femoral head with a more compact bone formation at the fracture site.

Conclusion:These results demonstrate the anti-osteoporoticand osteoregenerativeproperties of the aqueous extract of *C.rutidosperma*which could be partially due to the presence of Ursolic acid, phytol and Kaempferitrin detected in the extract.

Keywords: Osteoporosis, fracture, ovariectomy, histology, *Cleome rutidosperma*

INTRODUCTION

Osteoporosis is the most common bone disease affecting women especially after menopause causing increased bone fragility and fractures (Rachner et al., 2011; Sözen et al., 2017). The most frequent sites are vertebral fractures, wrist fractures, and fractures of the upper end of the femur(Chou et al., 2022). Osteoporotic fractures are responsible for significant medical impact and costs(Orcel & Funck-Brentano, 2011). It is estimated that 50% of women and 20% of men could suffer from an osteoporotic fracture at some point during their lifetime(Akesson et al., 2013), and the osteoporosis treatment causing aneconomic burden may rise up by 2040(LeBoff et al., 2022).Estrogen deprivation due to menopause remainsphysiological challenge to women as the skeletal protectiveeffects of estrogen are lost. The upswing in the radical oxygen species ROS leveland the release of pro-inflammatory cytokines adversely affect the survival and activity of osteoblasts by promotingformation and activity of osteoclasts(Mohamad et al., 2020). Although bone healing is aphysiological and spontaneous process, intervention andcreating ideal conditions can be useful for a rapid restorationof normal function and regeneration. Nowadays, variousmethods have been used tospeed up the healing process, including electrical stimulation (Gorustovich et al., 2002), pulsed ultrasound(Hadjiargyrou et al., 1998), andbone graft(Rumi et al. 2005).However, the use of these substances is not economicaland not available to the public and requires advancedhospital facilities. Therefore, alternative strategies using a non-pharmacological approach including dietary interventions are raising concerns(Shen et al., 2021).Several studies have been doneon bone healing using medicinal plants(Adhikari et al., 2017; Florence et al., 2017b; Florence et al., 2023).*C.rutidosperma* is a creeping herb of the family *Cleomacea*, which is used empirically in the treatment of arthritis, diabetes, diarrhoea, inflammation among others (Ghosh et al., 2019) and in the consolidation of bones in newborns, indicated by some traditional healers (personal communication). Tofurther exploit the therapeutic virtues of this plant, the present work aimed at investigating the osteoformative effects of the aqueous extract of

C.rutidosperma on osteoporotic fractures induced in female Wistarrats.

MATERIALS AND METHODS

Plant material:

The whole plant of *C.rutidosperma* was collected atNgoaEkele, in the Center region of Cameroon. The plant was authenticated at the Cameroon NationalHerbarium in comparison with the specimenvoucher N°29482 HNC.The whole fresh plant was cleaned,cut into pieces,and dried under a shade at roomtemperature.The extract was preparedaccording to the recommendations of thetraditional healer.The extract was obtained by maceration, introducing 100 g of *C.rutidosperma* powder in one liter of water for48hours, then, the mixture was filtered with the Wathmanfilter paper n°1and the filtrate was lyophilized.

Determination of mineral contentin the plant:

The total phosphorus content was determined by the"molybdovanadate" method. The determination of calcium and magnesium was carried out by the colorimetric method withcalmagite(Florence et al., 2017a; Gindler & Heth, 1971).

Quantitative analysis of Cleome rutidosperma aqueous extract:

The identification of the different groups of compounds of the aqueous extract of *C. rutidosperma* were determined using phytochemical tests based on colouring reactions and chromatographic analyses(El-Haoud et al., 2018).

LC/ESI-MS analysis of the aqueous extract of Cleome rutidosperma:

Liquid Chromatography Electrospray Ionization Mass Spectrometry (LC/ESI-MS)experiments were performed on a system consisting of a Waters Alliancemodel 2690 separation module and model 996 photodiode array detector(Waters, Eschborn, Germany) controlled with a Compaq AP200 work station coupled toa Micromass model ZMD mass detector (Micromass, Altrincham,Cheshire,UK). Thesamples were automatically injected into a Watersnarrow bore Nova-PakcolumnC18(2.1150mm,60Å pore size,3.5lm particle size). The elution was carried out with solventsA(0.1%TFA/ H2O) and B (60% acetonitrile/0.1% TFA/H2O) at a flow rate of 0.4 mL/minusing a linear gradient from 5% to 95%B in 30 min. The condition used for massspectrometry measurements was a positive ESI. The compounds were detected on theSciFinder database and identified by the values of their m/z ratio. In the view of the literature, this planthas already been the subject of several studies and all the detected peaks corresponded to already known compounds. However, a few ultra minoritypeakspresented unknown compounds.

Animals:

Female rats weighing 120 ± 20g were used in the present study. They were obtained from the animal house of the Faculty of Sciences at the University of Yaoundé I (Cameroon). Animals were submitted to the standard diet established in the laboratory and they received water *ad libitum*.The study was carried out with the authorization of the Cameroon National Ethical Committee (Ref n°. FW-IRB00001954).

Ovariectomy procedure:

Ovariectomy consistedofthe bilateral removal of the ovaries using the methodology described bySotiriadou et al. (2003) with slight modification. Briefly, twenty-five rats were anesthetized by intraperitoneal injection of valium (Diazepam, 10 mg/kg) and sleep was prolonged by Ketamine (30 mg/kg). Then the animals were placed in aprone position on a cork plate; a part of the lower back of the animals was disinfected with cotton soaked in 95° alcohol. Using forceps and scissors, a longitudinal incision was made on the waxed part. The uterine horns were removed from each ovary through an incision made between the uterine horn and the ovary. After this operation, the incision made on the back was closed with stitches. The animals were then treated with betadine and penicillin for 7 days to avoid infection. For the sham group (normal control), five animals underwent the entire ovariectomy procedure except that the ovaries were not removed.

Fracture induction:

Eighty-four days after ovariectomy, twenty-fiveovariectomized rats (OVX) were subjected to femoral fracture.The rats were anesthetized by intraperitoneal injection of valium at 10 mg/kg and ketamine at 30 mg/kg. An incision was made on the skin of the thigh, the muscle mass was removed, and the femur was exposed. A hole with a diameter of 1.5 mm was made on the distal end of the femur using an electric drill(Ngueguim et al., 2013). Then the opening was closed with stitches. The wound was treated with betadine and penicillin every day until healing.

Experimental design:

Ovariectomized fractured rats were divided into five groups of six rats each and treated as follows:

A negative control group of Ovx-fractured animals, treated with distilled water 10 mL/kg (OVX-F);

A positive control group of Ovx-fractured animals, treated with estradiolvalerate(E2V) (1mg/kg);

3 groups of Ovx-fractured animals treated with the plant extract at the dose of 75 (*C. rutidosperma* 75), 150 (*C. rutidosperma*100), and 300 mg/kg (*C. rutidosperma*300).

A normal control group (Sham), treated with distilled water 10mL/Kg was added. Substances were orally administereddaily for twenty- eight days. At the end of this experimental period, all animals were fasted for 12 hours in metabolic cages before sacrifice, and urine was collectedfor the determination of calcium and phosphorus. Animals were sacrificed by decapitation, the arterio-venous blood was centrifuged, and serum collected for some biochemical parameters (triglycerides, total cholesterol, HDL cholesterol, LDL-cholesterol, calcium, phosphorus, alkaline phosphatase); the homogenates of fractured bones were used for the determination of alkaline phosphatase activity, calcium,and phosphorus levels and some oxidative stressparameters(MDA, glutathione, nitrites, and catalase activity)and Tumornecrosis factor α (TNF-α), transforming growth factor β (TGFβ)and osteocalcin. The other part of the fractured femur was collected for radiography and histology, andthe radiographic score was determined.

Determination of the relative weight of some organs and the femoral density:

– Relative mass of organs was determined by using the fresh weight of the organs and calculated according toAkhtar et al. (2009)

Organ weight ratio = Femurweight (g) x100 / Body weight

– The femoral density was determined using the method of Emmanuell et al. (2021).
Femoral density = [femur wet weight (kg) × 1000 (kg/mm^3)/volume of femur (mm^3)]

Measurement of biochemical parameters:

In femoral tissue, lipid peroxidation was measured according to the method of Wilbur *et al*.(Wilbur et al., 1949). The method is based on the determination of malondialdehyde (MDA), the end product of lipid peroxidation. The catalase activity, reduced glutathione (GSH), and nitrites levels were evaluated as describedbySinha, 1972; Ellman, 1959 and Fermor*et al*., 2001 respectively(Ellman, 1959; Fermor et al., 2001; Sinha, 1972). The levels of phosphorus, calcium, the activity of alkaline phosphatase and some parameters of lipid profile were determined using Labkit kits.The serum level of osteocalcin was detected using ratEnzyme-linkedimmunosorbent assay (Sandwich-ELISA) kits from Elabscience. In the femur, the levels of transforming growth factor-β (TGF- β) and tumor Necrosis Factor-α (TNF- α) were determined using rat ELISA kits from Biomed.

Radiographic and histologicalanalyses:

Radiographs were taken to evaluate the femoral bone healing process. Bone formation was evaluated according to Lane and Sandhu method (Bigham et al., 2008)score as follow:

No evidence of bone formation 0
Bone formation occupying 25% of defect 1
Bone formation occupying 50% of defect 2
Bone formation occupying 75% of defect 3
Bone formation occupying 100% of defect 4

For histological analysis, sections of decalcified femurs fixed in 10% neutralbuffered formalin and embedded in paraffin wax weretaken using a microtome and stained with hematoxylin and eosin(Tsofack Ngueguim et al., 2023). The sections were mounted and observed forhistopathological changes.

Statistical analysis:

Statistical significance was determined by one-way ANOVA analysis of variance followed by the Turkey post-test using GraphPad Prism version 8.0 software. All data were expressed as mean ± SE and differences between means were considered significant at P<0.05.

RESULTS

Mineral content in the aqueous extract of C.rutidosperma:

The mineral contents of interest in µg/g of the aqueous extract of *C.rutidosperma*aresummarized in Table 1. The analysis of the mineral content of the plant extract showed the presence of calcium, phosphorus,and magnesium. Phosphorus was found to have priority (1100 µg/g) over calcium (50 µg/g) and magnesium (10 µg/g).

Table 1: Mineral content of the aqueous extract of *C.rutidosperma*

Minerals	**(µg/g)**
Calcium	**50**
Phosphore	**1100**
Magnesium	**10**

Quantitative phytochemical compositionof the aqueous extract of C. rutidosperma:

Phytochemical screening of the aqueous extract of *C.rutidosperma* revealed that the plant is rich in active substances. The various assays for flavonoids, tannins, alkaloids, saponins and polyphenols indicate a high content of polyphenols (1468.67± 120.00 µg EAG/g DM) follow by alkaloids (991.52 ± 98.10µg EQi/g DM), flavonoids (742.78 ± 12.70 µg EQ/g DM), then saponines (272.30 ± 22.80 (µg EDi/g DM) and by tannins (111.09 ± 6.78µg EAT/g DM) (Table 2).

Table 2: Quantitative phytochemical composition

Group of Compounds	**Average**
Flavonoids (µg EQ/g DM)	742.78±12.70
Tannins (µg EAT/g DM)	111.09 ± 6.78
Alkaloids (µg EQi/g DM)	991.52 ± 98,10
Saponines (µg EDi/g DM)	272.30 ± 22.80
Polyphenols (µg EAG/g DM)	1468.67120.00

Data points are means of triplicate experiments. DM : dry materials

LC/ESI-MS analysis:

Figure 1 and Table 3 summarize the major peaks and compounds respectively in the chemical profile of the aqueous extract of *C. rutidosperma*. Sixteen compounds were detected (Fig. 1) among which, seven were isolated.

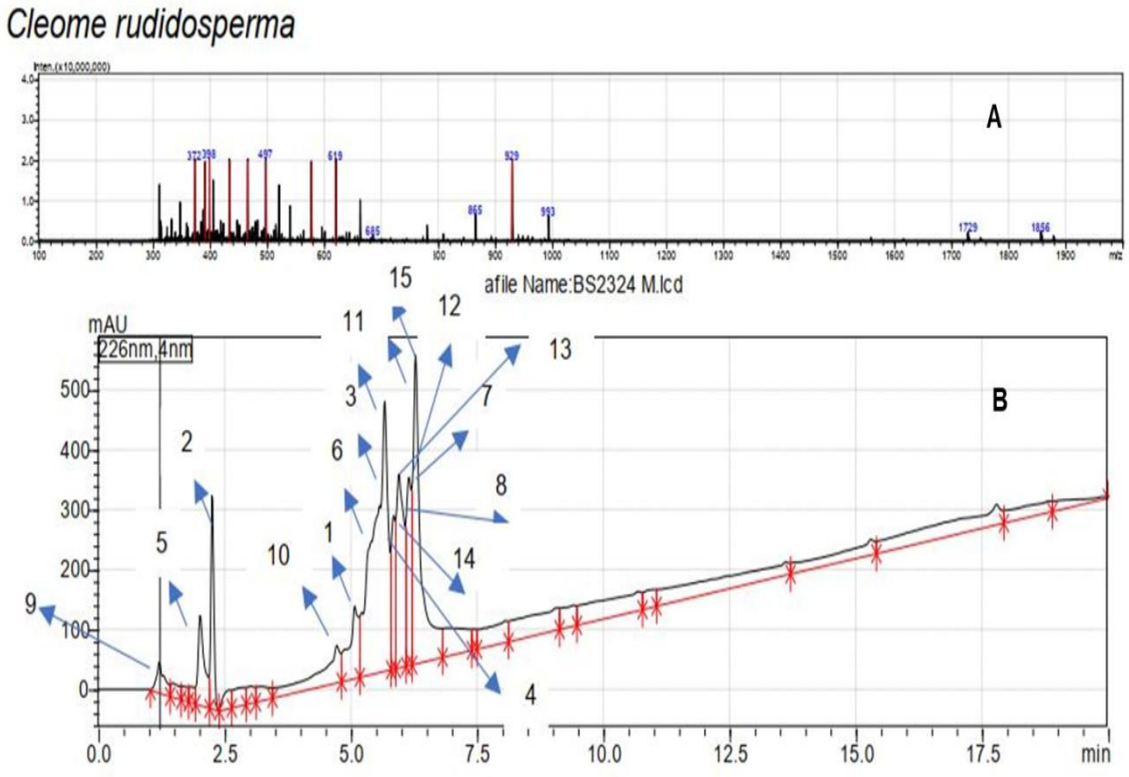

Figure 1: LC/ESI-MS profile of the aqueous extract of *C. rutidosperma*

A represents LC/ESI-MSprofile of the aqueous extract of *C. rutidosperma*and, B spectra of each detected compounds.

Table 3: Main signals exhibited in the LC/ESI-MS spectra of compounds detected in the aqueous extract of *C. rutidosperma*

Num	Detected compounds
1	(4R,7S,8R)-2-hydroxy-1,4,7,8-tetramethyl-17-methylene-15-oxatricyclo[9.3.2.14,8]heptadec-11-en-16-one.
2	(1E,5E,11E)-11-formyl-5-methyl-8-(prop-1-en-2-yl)cyclotetradeca-1,5,11-trienecarboxylic acid.
3	Cleomaldicacid
4	Paradoxenoicacid
5	Phytol
6	Methyl ester of 2,18-O-diacetyl-16-O-(3-hydroxy-3-methylglutaryl)-7 hydroperoxydolabella3,8(17)diene-2,16,18 triol
7	Brachycarpone
8	Deacetoxybrachycarpone
10	Amblyone
11	Ursolicacid
12	Quercetin 3-O-(2″acetyl)-glucoside
13	Visconoside B
14	Vincetoxicoside A
15	Kaempferitrin

Evolution of body weight:

The curves below show the weight evolution before and during the treatment of the ovariectomized-fractured animals(Fig. 2). Body weight gain was higher in ovariectomized females than in the sham group, and similar in ovariectomizedfemales with estradiol substitution and those who were treated with the aqueous extract of *C.rutidosperma*.

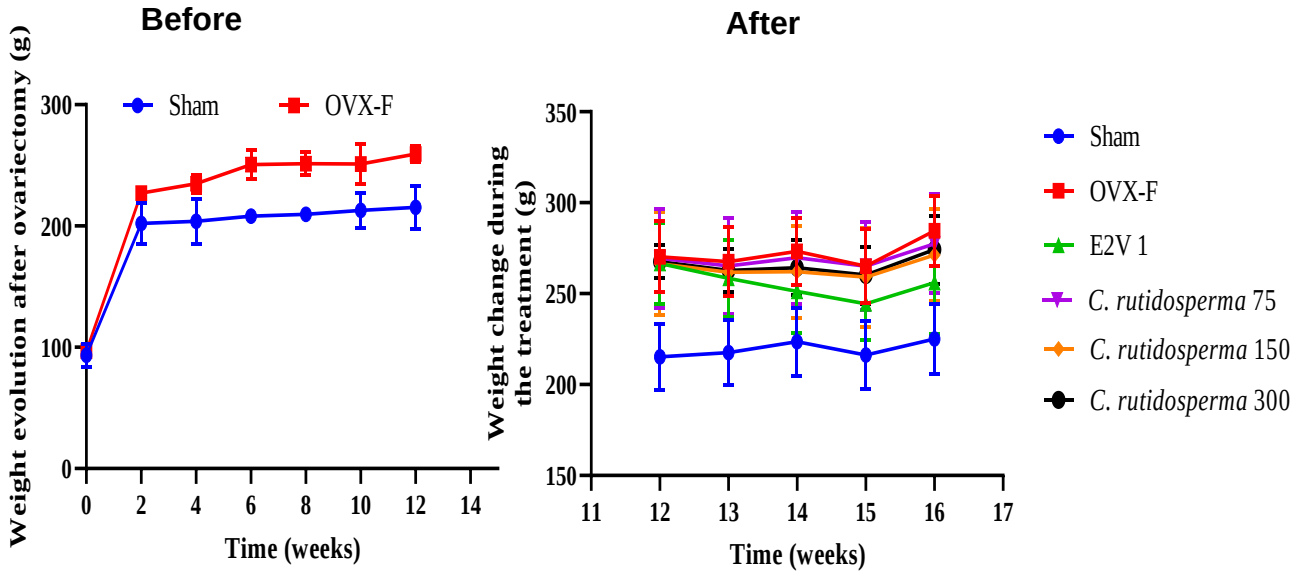

Figure 2: Weight evolution of ovariectomized animals before and after treatment.

Each point represents the mean ± SEM (n=5). **Sham**: normal control animals with simulated surgery treated with distilled water (10mL/kg); **OVX-F**: negative control ovariectomized-fractured animals treated with distilled water (10mL/kg); **E2V 1**: positive control ovariectomized-fractured animals treated with estradiolvalerate (1mg/kg); *C. rutidosperma*75, 150, 300: ovariectomized-fractured animals treated with plant extract at the dose 75 mg/kg**;**150 mg/kg; 300 mg/kg.

Effect on relative weight of some organs and bone density:

In ovariectomized-fractured rats, it has been observed a non-significant decrease in femur mass in the negative control group compared to the normal Sham control. However, the different treatments had no impact on femur mass. Ovariectomy followed by the fracture resulted in a significant decrease ($p<0.001$) in bone density, vagina, and uterus weight compared to the normal sham control animals. The plant extract at the dose of 150 mg/kg significantly increased ($p<0.05$) bone density compared to the negative control group and the treatment with estradiolvalerate significantly increase the uterus weight (Table 4).

Table 4: Effects of the aqueous extract of *C.rutidosperma* on relative weight of some organs and bone density in ovariectomized-fractured female rats.

Parameters	**Sham**	**OVX-F**	**E2V 1**	***C. rutidosperma*75**	***C. rutidosperma*150**	***C. rutidosperma*300**
Weight of fractured femur(g)	0.34±0.01	0.30±0.01	0.33±0.01	0.34±0.02	0.32±0.01	0.30±0.02
Bone density (mg/mL)	3.45±0.37	2.17±0.01$^{\gamma}$	2.19±0.10	2.48±0.04	2.93±0.11^{a}	2.43±0.03
Mass of vagin (g)	0.09±0.01	0.05±0.01$^{\gamma}$	0.06±0.02	0.05±0.03	0.05±0.01	0.06±0.01
Mass of uterus (g)	0.23±0.01	0.02±0.01$^{\gamma}$	0.10±0.01^{c}	0.03±0.01	0.03±0.01	0.02±0.01

Each value represents the mean ± SEM (n=5). $^{\gamma}P<0.001$: significant difference from Sham normal control group; $^{a}P<0.05$; $^{c}P<0.001$: significant difference from negative control group; Sham: normal control animals with simulated surgery treated with distilled water (10mL/kg); OVX-F: negative control ovariectomized-fractured animals treated with distilled water (10mL/kg); E2V: positive control ovariectomized-fractured animals treated with estradiolvalerate (1mg/kg); *C. rutidosperma* 75, 150, 300: ovariectomized-fractured animals treated with plant extract at the dose 75 mg/kg,150 mg/kg or 300 mg/kg.

Effects of the aqueous extract of C.rutidosperma on some lipid profiles:

Table 5presents the effects of the aqueous extract of *C. rutidosperma* on lipid profile parameters and atherogenic index. Ovariectomy associated with fracture resulted in a significant increase ($p<0.001$) in LDL cholesterol, triglyceride, and atherogenic index levels while a significant decrease ($p<0.001$) in HDL cholesterol levels was observed compared to the sham group. Treatment with aqueous extract of *C. rutidosperma* at the doses of 75 mg/kg, 150 mg/kg and 300 mg/kg, as well as treatment with oestradiolvalerate, significantly reduced ($p<0.001$) LDL cholesterol, triglyceride and atherogenic index levels and increased ($p<0.001$) HDL cholesterol levels compared to OVX-F group.

Table 5: Effects of the aqueous extract of *C.rutidosperma* on some lipid profileparameters

Parameters	Sham	OVX-F	E2V 1	*C. rutidosperma* 75	*C. rutidosperma* 150	*C. rutidosperma* 300
TC (mg/dL)	91.00±3.73	105.00±5.82	110.00 ±5.18	90±3.71	85.50 ±3.31	107±4.80
HDL-C (mg/dL)	63.62±1.42	41.44±1.31 $^{\gamma}$	81.80±1.42 ^{c}	64.40±3.90 ^{c}	73.82±3.23 ^{c}	73.50 ±2.71 ^{c}
LDL-C (mg/dL)	18.70±2.29	47.20±5.03 $^{\gamma}$	26.70±2.40^{b}	24.50±2.52^{b}	8.10±3.66^{c}	31.70±1.51$^{\alpha}$
TG (mg/dL)	36.46±1.55	54.70±3.91 $^{\gamma}$	32.82±1.63 ^{c}	40.60±3.74 ^{b}	35.25±2.23 ^{c}	27.33±1.47 ^{c}
AI	1.20±0.14	2.54±0.11$^{\gamma}$	1.35±0.06^{c}	1.56±0.07^{c}	1.16±0.03^{c}	1.55±0.20^{c}

Each value represents the mean±SEM (n=5).$^{\alpha}$ P<0.05;$^{\gamma}$P<0.001: significant difference from Sham normal control;^{b} P<0.01; ^{c} P<0.001: significant difference from **OVX-F** lot. **Sham**: normal control animals with simulated surgery treated with distilled water (10mL/kg);**OVX-F**: negative control ovariectomized-fractured animals treated with distilled water (10mL/kg); **E2V 1**: positive control ovariectomized-fractured animals treated with estradiolvalerate (1mg/kg); ***C. rutidosperma* 75, 150, 300**: ovariectomized-fractured animals treated with plant extract at the dose 75 mg/kg**;**150 mg/kg; 300 mg/kg;**TC**: total cholesterol;**HDL-C**: high-density lipoprotein cholesterol;**LDL-C**: low-density lipoprotein cholesterol;**TG**: triglycerides; **AI**:atherogenic index.

Effects of the aqueous extract of C.rutidospermaon some biochemicalparameters:

Ovariectomy associated withfracture induced a significant increase (p<0.01) in serum alkaline phosphatase activity while serum phosphorus decreased (p<0.05) in theOVX-F control group compared to thesham group (Table 6). The plant extract at all doses significantly decreased (p<0.01) the alkaline phosphatase activity but did not affect serum phosphorus as compared to the OVX-F group. Treatment with estradiolvalerate significantly p<0.05) decreased serum alkaline phosphatase activity. OVX-Fractured rats exhibited a significant (p<0.01) decrease in bone ALP activity, calcium, and phosphorus concentrations. The administration of the extract led to a significant increase in ALP activity and bone phosphorus concentration at all doses (p<0.05-p<0.01) while bone calcium significantly increased only at the doses of 75 mg/kg (p<0.01) and 150mg/kg (p<0.05). There were also a significant increases in urinary calcium and phosphorus of OVX-F rats compared to the sham group. The administration of the plant extract induced at all doses a significant decrease (p<0.05-p<0.01) in urinary phosphorus while, only the dose of 150 m/kg of the plant extract significantly decreased (p<0.05) urinary calcium. Treatment with estradiolvalerate induced a significant decrease (p<0.05) in ALP activity and urinary calcium while bone phosphorus significantly increases (p<0.01) as compared to the OVX-fractured group.

Table 6:Effects of the aqueous extract of *C.rutidosperma* on some biochemical parameters of bone metabolism

Parameters	Sham	OVX-F	E2V 1	*C. rutidosperma* 75	*C. rutidosperma* 150	*C. rutidosperma* 300
Serum Alkaline phosphatase (U/I)	78.50±2.31	120.01±4.52 $^{\beta}$	90.93±6.85^{a}	80.34±3.97^{b}	81.30±7.08^{b}	76.57±7.23^{b}
Serumcalcium (mg/dL)	35.95±29.04	35.85±2.66	36.51± 0.82	34.43±0.42	38.46±3.07	33.52±2.72
Serumphosphorus (mg/dL)	7.80±0.5	6.30 ±0.20$^{\alpha}$	7.10 ±0.30	7.50 ±0.40	6.50 ±0.30	6.30 ±0.20
Bone Alkaline phosphatase (U/I)	4153.00±52.51	2469.03±32.31$^{\gamma}$	3053.07±139.04	3168.09±113.01^{a}	3584.02±22.013^{c}	3548.06±192.01^{c}
Bone calcium (mg/dL)	145.04±6.71	86.72±2.33$^{\gamma}$	104.01±7.43	126.02±7.22^{b}	117.01±1.72^{a}	107.04±2.13
Bonephosphorus (mg/dL)	53.85±0.94	41.73±1.62$^{\gamma}$	52.23±1.62^{b}	50.81±1.15^{a}	50.60±1.10^{a}	58.64±1.70^{c}
Urinary calcium (mg/dL)	88.20±2.92	124.03±8.25$^{\beta}$	93.01±5.57 ^{a}	109.03±7.54	92.10±7.31^{a}	97.12±3.14
Urinaryphosphorus (mg/dL)	24.85±1.21	33.33±0.82$^{\beta}$	32.84±0.31	23.50±2.60^{b}	22.82±1.72^{c}	26.23±0.46^{a}

Each value represents the mean±SEM (n=5). $^{\alpha}$P<0.05; $^{\beta}$P<0.01; $^{\gamma}$P<0.001: significant difference from Sham normal control lot; ^{a}P<0.05; ^{b}P<0.01; ^{c}P<0.001: significant difference from negative control lot. **Sham**: normal control animals with simulated surgery treated with distilled water (10mL/kg); **OVX-F**: negative control ovariectomized-fractured animals treated with distilled water (10mL/kg); **E2V 1**: positive control ovariectomized-fractured animals treated with estradiolvalerate (1mg/kg); ***C. rutidosperma* 75, 150, 300**: ovariectomized-fractured animals treated with plant extract at the dose 75 mg/kg**;**150 mg/kg; 300 mg/kg.

Effects of the aqueous extract of C.rutidosperma on some parameters of oxidative stress:

Figure 3 presents the effects of the aqueous extract of *C. rutidosperma* on some parameters of oxidative stress in the bone of OVX-F rats. Ovariectomy associated with fracture resulted in a significant increase (p<0.001) in bone malondialdehyde and a significant decrease in GSH (p<0.001) and nitrites (p<0.05) concentrations compared to the sham control group. Treatment with the plant extract at the dose of 75 mg/kg induced a significant (p<0.001) decrease in MDA concentration while the nitrites

concentration increased. The treatment with the extract at the dose of 150 mg/kg increases GSH ($p<0.05$) and nitrites ($p<0.001$) concentrations, with a decrease in MDA ($p<0.01$) level. At the dose of 300 mg/kg, the extract showed a decrease in MDA level and an increase in GSH ($p<0.001$) and nitrites levels compared to the OVX-F group. Compared to the OVX-F group the extract at a dose of 150 mg/kg increased catalase activity ($p<0.05$). Estradiolvalerate treatment also significantly increased GHS ($p<0.001$), nitrite ($p<0.01$) level, and catalase ($p<0.001$) activity however decreased MDA concentration ($p<0.001$) as compared to OVX-F group.

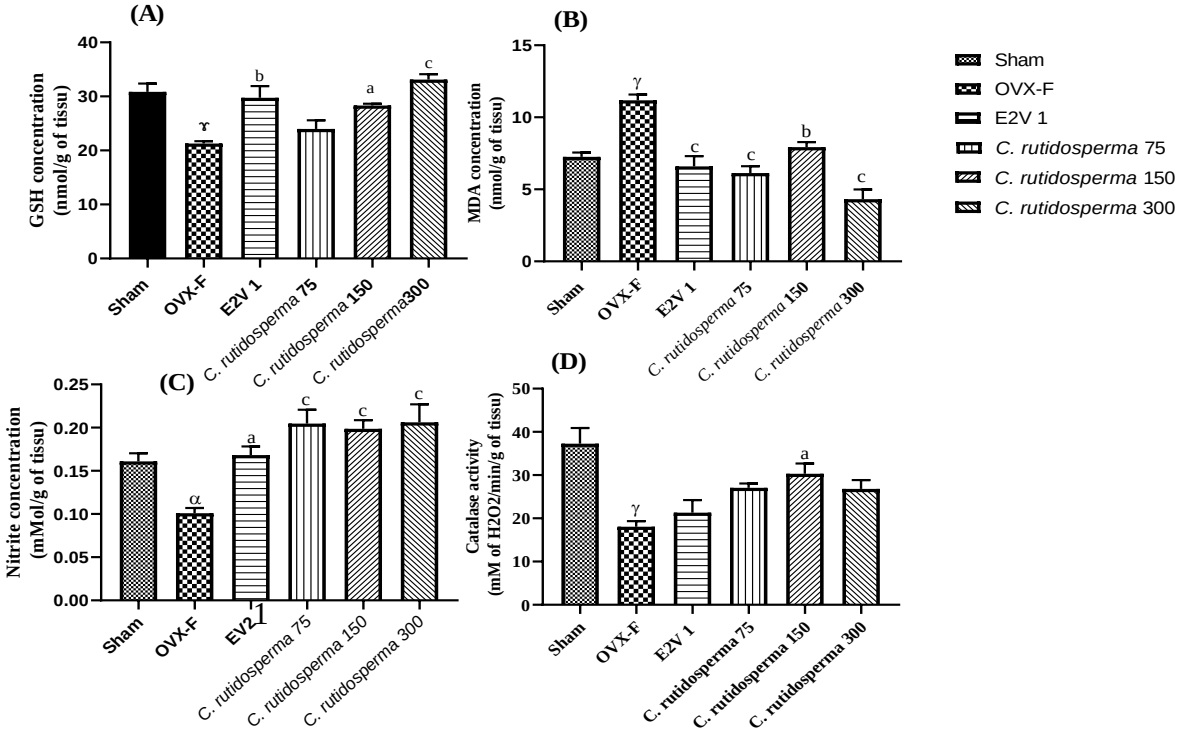

Figure3: Effects of the aqueous extract of *C.rutidosperma* on GSH (A), MDA (B), nitrites (C) concentration and catalase (D) activity in OVX-F rats

Each bar represents the mean ± SEM (n=5). $^{\alpha}P<0.05$; $^{\gamma}P<0.001$: significant difference from normal control **Sham**; $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$: significant difference from negative control; **Sham**: normal control animals with simulated surgery treated with distilled water (10mL/kg);**OVX-F**: negative control ovariectomized-fractured animals treated with distilled water (10mL/kg); **E2V 1**: positive control ovariectomized-fractured animals treated with estradiolvalerate (1mg/kg); ***C. rutidosperma*75, 150, 300**: ovariectomized-fractured animals treated with plant extract at the dose 75 mg/kg**;**150 mg/kg; 300 mg/kg.

Effects of the aqueous extract of C.rutidospermaon the levels of TGF-β, TNF-α, and osteocalcin:

Ovariectomy and fracture in Figure 4resulted in significantly decreased levels of TGF-β ($p<0.001$)and an increase of TNF-α ($p<0.001$) level in bone in comparison with theshamgroup. The administration of the plant extract significantly increased TGF-β concentration at the doses of 75 mg/kg ($p<0.01$), 150 mg/kg ($p<0.05$), and 300 mg/kg ($p<0.01$) compared to OVX-F rats. Following the four weeks treatment period with the extract at the doses of 75 mg/kg and 150 mg/kg, the TNF-α level significantly decreased ($p<0.001$). However, the plant extract at the dose of 300 mg/kg failed to significantly reduce the level of TNF-α. Estradiolvalerate at a dose of 1mg/kg decreased ($p<0.001$) the TNF-α level in comparison with the OVX-F group. Ovariectomized and fractured rats exhibited a significant decrease in osteocalcin levels compared to the sham group. On the contrary, the administration of the extract for four weeks significantly increased osteocalcin concentration at the doses of 75 mg/kg ($p<0.001$), 150 mg/kg ($p<0.01$), and 300 mg/kg ($p<0.001$). Similar result was observed when ovariectomized and fractured animals were treated with estradiolvalerate at a dose of 1mg/kg.

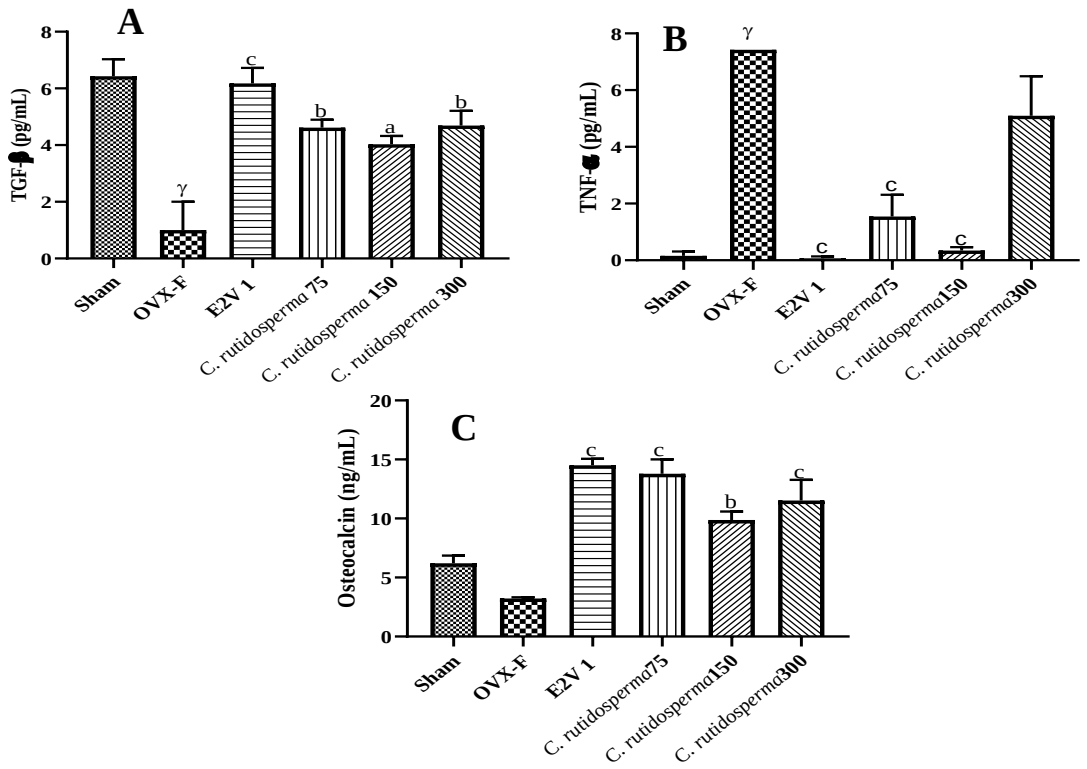

Figure4: Effects of aqueous extract of *C.rutidosperma* onTGF-β (A), TNF-α (B) and osteocalcin(C) levels

Each bar represents the mean ± SEM (n=5). $^{\gamma}$ P<0.001: significant difference from normal control **Sham**; ^{a} P<0.05; ^{b} P<0.01; ^{c} P<0.001: significant difference from negative control; **Sham**: normal control animals with simulated surgery treated with distilled water (10mL/kg);**OVX-F**: negative control ovariectomized-fractured animals treated with distilled water (10mL/kg); **E2V 1**: positive control ovariectomized-fractured animals treated with estradiolvalerate(1mg/kg);***C. rutidosperma* 75, 150, 300**: ovariectomized-fractured animals treated with plant extract at the dose 75 mg/kg**;**150 mg/kg; 300 mg/kg.

Radiographic and histological analysis:

The effect of *C. rutidosperma* on femoral head and bone callus formation was summarized in Figure5. OVX-F rats showed less than 25% bone formation compared to a sham group (Table 7). The treatment of ovariectomized-fractured rats resulted in 75%, 50%, and 25% of bone formation respectively at the doses of 75, 150, and 300 mg/kg compared to OVX-F. As presented in Figure 5, ovariectomyinduced bone resorption with a defect in bone regeneration at the fracture site.The femoral head showed numerous large bone resorption accompanied by several areas of resorptionlacunae (Fig. 5A). In the fracture zone of these animals, bone consolidation was still at the embryonic stage characterized by a loose arrangement, with disorganised collagen fibres compared with the normal sham control (Fig.5B). The administration of the plant extract at the doses of 300mg/kg and 150 mg/kg reduced resorption lacunae and improved bone consolidation whatever incomplete compared to ovariectomized-fractured control.The extract treatment at a dose of 75 mg/kg induced bone formation with a compact callus with the onset of trabecular filling at the femoral heads and reorganization of the compact bone in the fracture site. Estradiolvalerate treatment resulted in 75% bone formation with a reduction of bone resorption and slight bone consolidation compared to OVX-F.

Table 7: Effect of the plant extract of *C.rutidosperma* on the bone radiography assessment

	Median (min-max)					
Group	**Sham**	**OVX-F**	**E2V 1**	***C. rutidosperma* 75**	***C. rutidosperma* 150**	***C. rutidosperma* 300**
Score	4 (4-4)	1 (0-1) $^{\gamma}$	3 (2-3) ^{c}	3 (3-4) ^{c}	2 (1-3) ^{b}	1 (0-1)
Bone formation (%)	100%	>25%	75%	75%	50%	> 25%

Each value represents the mean ± SEM (n=5). $^{\gamma}$ P<0.001: significant difference from normal control **Sham**; ^{b} P<0.01; ^{c} P<0.001: significant difference from negative control. **Sham**: normal control animals with simulated surgery treated with distilled water (10mL/kg); **OVX-F**: negative control ovariectomized-fractured animals treated with distilled water (10mL/kg); **E2V 1**: positive control ovariectomized-fractured animals treated with estradiolvalerate (1mg/kg); ***C. rutidosperma* 75, 150, 300**: ovariectomized-fractured animals treated with plant extract at the dose 75 mg/kg**;**150 mg/kg; 300 mg/kg.

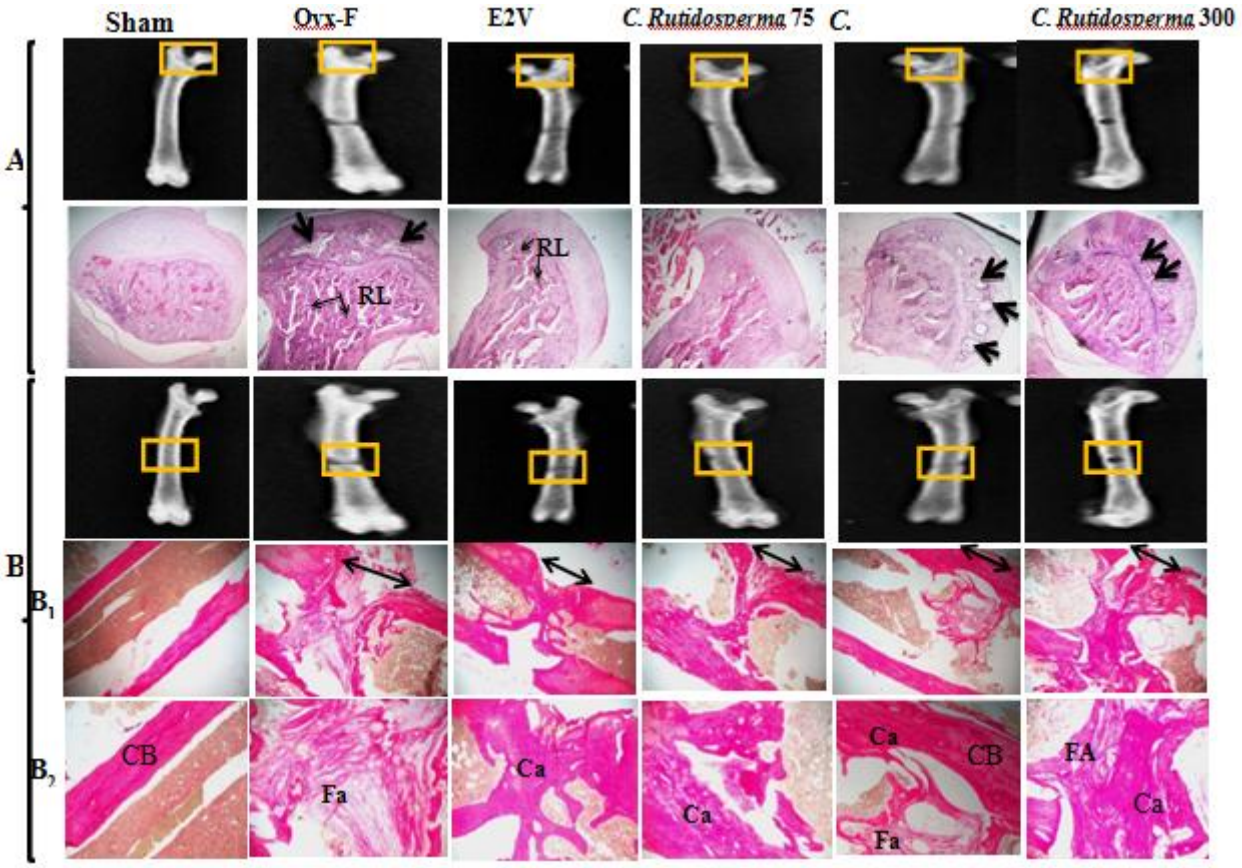

Figure 5: Radiographyand microphotographs of the femoral head (A) and fracture site (B) of ovariectomized-fractured female rats after treatment

Sham: normal control animals with simulated surgery treated with distilled water (10mL/kg); **OVX-F**: negative control ovariectomized-fractured animals treated with distilled water (10mL/kg); **E2V**: positive control ovariectomized-fractured animals treated with estradiolvalerate (1mg/kg); ***C. rutidosperma* 75, 150, 300**: ovariectomized-fractured animals treated with plant extract at the dose 75 mg/kg**;**150 mg/kg; 300 mg/kg. **A: Femoral head**,Hematoxylin-Eosine stain (x250). **B: fracture site,**Picro Sirius stain (x100 for $\mathbf{B_1}$ and x250 for $\mathbf{B_2}$).**RL**: Resorption Lacunae, **Single arrow**: bone resorption on femoral head, **double arrow**: fracture site, **CB**: Cortical Bone, **Fa**: Fibrous aspect, **Ca**: compact aspect.

DISCUSSION

Osteoporosis is the most common bone disease affecting women especially after menopause, and causes increased bone fragility and fractures(Ji & Yu, 2015).In menopause, bone formation decreases and bone resorption exceeds, subsequently, the risk of fracture increases. Fractures are the most common problem associated with osteoporosis and despite advances in the prevention and treatment of osteoporosis the number of fractures continue to increase(Lems & Raterman, 2017). The purpose of the study was to investigate the effects of aqueous extract of *C. rutidosperma* on osteoporotic fractures. Ovariectomized and fractured rats were characterized by a dramatic decrease in uterus and vagina weight compared to thesham group. The changes in organ weight in these rats might be due to the atrophy of the endometrium and vaginal epithelium resulting from the lack of hormones secreted by the ovaries. Various studies have shown thatestrogensstrongly stimulate cell proliferation in the uterine endometrium(Groothuis et al., 2007; Yu et al., 2022). In addition, the significant increase in uterine mass in the positive control group confirms the estrogenic activity of estradiolvalerate. The extract did not affect the vagina and uterus implying that the extract could not mimic the estrogen like effect. The obtained resultsrevealed that estrogen insufficiency caused hypertriglyceridemia as well as increased LDL-C and decreased HDL-C levels. It has been shown that high levels of LDL-cholesterol may also cause changes in bone alterations in bone tissue, such as inhibition of osteoclastic activity, a decrease in bone remodeling, and a reduction in bone mass(Choukroun et al., 2014). The treatment with the plant extract and estradiolvalerate decreased the parameters of the lipid profile compared with the negative control group. This result may attribute to hypolipidemic properties to the aqueous extract of *C.rutidosperma*. This result may be due to the action of flavonoids and alkaloids present in the aqueous extract of *C. rutidosperma* which have been proved to reduce TG and cholesterol levels in the blood and increased serum HDL-C levels(He et al., 2016).

Osteoporosis is characterized by a drop in bone density, confirmed in the present study by the decrease in mineral density of OVX-F rats. These results could be explained by the imbalance between bone formation and bone resorption process due toestrogen deficiency following menopause (Møller et al., 2020).*C. rutidosperma* extract at the dose of 150 mg/kg, significantly increased bone density inovariectomized and fractured animals compared to OVX-Fgroup. This could be due to the minerals present in the plant extract, which could be fixed on the bone matrix, thus

increasing the density of the bone and consequently, repairing the fracture. Indeed, calcium supplementation is likely to increase bone density(Winzenberg et al., 2006).

Alkaline phosphatase (ALP) in bone is an indicator of bone formation and a higher serum level of this enzyme shows faster maturation and more activity of osteoblasts(Vimalraj, 2020). Alkaline phosphatase is secreted by osteoblasts and plays an important role in bone healing(Blair et al., 2017). This enzyme triggers the mineralization of the osteoid by increasing the local concentration of calcium phosphate(Vimalraj, 2020). In this study, alkaline phosphatase activity was significantly increased in serum and decreased in bone in theOVX-F group. Indeed, the higher ALP in serum and the lower in bone observed may reflect an increased bone turnover rate rather than simply increased bone formation and thus may be associated with an increased risk of bone fracture(Atalay et al., 2012). Treatment with estradiolvalerate as well as the aqueous extract of *C. rutidosperma* had a positive effect on serum and bone phosphatase activity. This result is similar to that ofHasib et al. (2020). It could be a notion that the fracture healing got into the catabolic stage and the presence of flavonoids in the plant extract which stimulate osteoblastic activity to significantly increased ALP activity.

Indeed, the ALP secreted in the bone by osteoblasts accelerates the process of mineralization either by increasing the local concentration of inorganic phosphate or activating the collagen fibers to induce the deposition of calcium salts and involved in bone formation and healing of thefracture(Vimalraj, 2020)(Prouillet et al., 2004). The important role of Ursolic acid, phytol and Kaempferitrinhas been demonstrated in bone metabolism. Phytol suppresses osteoclast differentiation(Islam et al., 2020), ursolic acid on osteoclastogenesis and titanium particle-induced osteolysis are mediated primarily via suppression of NF-κB signalling, andKaempferitrin prevents bone loss and increases bone formation in ovariectomized rats(Ma et al., 2015).These compoundscould react together to improve bone microarchitecture andaccelerate bone formation in ovariectomized-fractured rats.

Calcium and phosphorus are the main minerals in bone, present in the form of calciumhydroxyapatite crystals, whichplay an important role in regulating the elastic stiffness and tensile strength of bone (Adhikari et al., 2017). In this study, the absence of any significant change in serum calcium levels shows that hemostatic mechanisms maintain the serum calcium levels even after ovariectomy(Shirwaikar et al., 2003). In the bone, phosphorus and calcium levels were significantly decreased in OVX-F rats. According toElkomy & Elsaid (2015), ovarian hormone deficiency following ovariectomy, is marked by the reduction of intestinal calcium absorption and may contribute to the accompanying bone loss (Elkomy & Elsaid, 2015).

This result coincides with that obtained. The obtained results suggested that, osteoporosis is a kind of chronic disease related to calcium reduction(Shen et al., 2021).The plant extract at the doses of 75 mg/kg and 150 mg/kg significantly increased the bone calcium and phosphorus levels suggesting the involvement of calcium and phosphorus within the plant contributes to bone formation and thanks to flavonoid which was shown to be able to prevent the reduction of calcium and phosphorus in bones (Oršolić et al., 2014). These micronutrients could be absorbed at the intestinal level and then reabsorbed in the tubular nephron, justifying the decrease of their urinary excretion and their deposition at the fracturedsite. This result corroborates that ofHasib et al. (2020), who showed that honey increases intestinal absorption of calcium by decreasing luminal pH. On the contrary, OVX-F rats showed a significant increase in urinary calcium confirming bone defect(Nordin et al., 1991). Moreover, polyphenols are known to significantly lower the excretion of Ca in the urine of rats (C. L. Shen et al., 2008).

Oxidative stress occurs in osteoporosis associated with fracture as evidenced by the increase in MDA level, the decrease in GSH, nitrite concentrations, and catalase activity. This stress is known to activate the differentiation of osteoclast and inhibit osteoblast differentiation(Agidigbi & Kim, 2019; Wauquier et al., 2009)a causative factor of osteoporosis-induced bone fragility. The plant extract at all doses reduced oxidative stress by improving theantioxidant status of rats, probably due to the antioxidant effect of the extract. Phytochemical analysis of the plant revealed bioactive components such as flavonoids and tannins(Bose et al., 2008) which possess beneficial antioxidant activity in bone tissue repairby their ability to trap free radicals(Ghosh et al., 2019; Gülçin et al., 2010; Mpondo et al., 2012; Raggatt & Partridge, 2010) Indeed, phytol and Kaempferitrin are respectively terpen and flavonoiddetected within the extract. These compounds can suppress reactive oxygen and/or nitrogen species produced by cellular stress (Islam et al., 2018; Jiang et al., 2018).

TNF-α is an important pro-inflammatory cytokine playing a crucial role in diseases, such as tumors and inflammation(Jang et al., 2021). It was shown that TNF-α can cause stem cell dysfunction during chronic inflammation and can inhibit osteogenic differentiation of bone marrow mesenchymal stem cells. In this study, the high level of TNF-α intheOVX-F group is a sign of chronic inflammation due to estrogen deficiency and would participate in the development of osteoporosis and consequently fragility fracture(Weitzmann & Pacifici, 2006). The significant decrease of TNF-α levels in ovariectomized-fractured animals treated with*C. rutidosperma* justifies the anti-inflammatory properties of the substances probably due to the anti-inflammatory effect of phytol(Islam et al., 2020) and Kaempferitrin(Ramos-Hernández et al., 2017). Indeed, polyphenols found in the plant extract are known to be capabledownregulatinginflammatory mediators, including osteoclast differentiation and activity by the osteoblast production of several signaling proteins, includingTNF-α(Leotoing et al., 2014). The significant increase in TGF-β levels in the test groups confirms this anti-inflammatory effect of the aqueous extract noted (Ghosh et al., 2019). Moreover, knowing that TGF-β regulates osteoblasts and chondrocytes differentiation, proliferation, and maturation implies bone formation(Wu et al., 2016), we could suggestthat the increase of TGF-β in extract-treated groups demonstrates the increase in bone formation in ovariectomized-fractured animals.Overexpression of TGF-β

in mice increases bone formation, probably through its direct stimulatory effects on osteoblast function(Erlebacher et al., 1998),and allows estradiolvalerate to exercise its protective role (Koohpeyma et al., 2020).

Osteocalcin is a vitamin K-dependent calcium-binding protein synthesized by osteoblasts during matrix mineralization. It is then incorporated into bone, but a fraction is directly secreted into the blood where it can be measured(Di Medio & Brandi, 2021).Osteocalcin is a sensitive indicator of boneturnover, specifically indicatingbone formation in various metabolic bone diseases(Moser & van der Eerden, 2018; Price et al., 1980).In this study, the OVX-F group showed a decrease in osteocalcin concentration attesting to bone impairment. This result was strengthened by the non-consolidation of the fracture area shown by the radiological score and, bone resorption at the femoral head. The high level of osteocalcin in the different groups treated with plant extract and estradiolvaleratemight be due to the presence of polyphenols in the plant extract, which has proved to increase serum osteocalcin levels and may stabilize lumbar spine bone mineral density (Filip et al., 2015).This would be at the origin of the bone consolidation observed on radiographic images and histological sectionsof the fracture zone, by the welding of this area. In the femur heads, there was a reduction of resorptionlacunae filled by bone formation. Indeed, some studies led to the hypothesis that osteocalcin exerts a mechanical function within the bone matrix. As a result of its ability to tightly bind hydroxyapatite and form a complex with collagen through the matrix protein osteopontin, osteocalcin was proposed as means to bridge the matrix and mineral fractions of bone tissue. Such an arrangement is compatible with the formation of dilatational bands that are seen when bone fractures(Hauschka & Carr, 1982; Hoang et al., 2003; Ritter et al., 1992). This effect could be also due to the minerals contained in the plant, with the anti-inflammatory and antioxidant properties presumed above(Ghosh et al., 2019; Raggatt & Partridge, 2010).

CONCLUSION

The aqueous extract of *C.rutidosperma* showed an improvementof lipid profile, a reduction of oxidativestress characterized by decreasing of MDA level while nitrite, GSH levels, and catalase activity decreasing. Moreover, the plant extract reduced inflammation by decreasing TNF-α concentration.Also, the extract demonstrated an osteoformative effect in ovariectomy-induced osteoporosis and bone fractured rat model. This was achieved by the increase in osteocalcin and TGF-β concentrations accompanied by a compact bone formation at the fracture site with a disappearance of the bone resorption areaof the femoral head. These findings provide the efficacity ofthe aqueous extract of *Cleome rutidosperma*for a probable future drug development for menopause induced bone fragility and fracture. However; more research are required to shed light on the mechanism through which the plant extract insures osteoformation.

Abbreviations:

ALP: Alkaline phosphatase
EV2:Estradiolvalerate
GSH: Glutathione
MDA: Malondialdhehyde
OC: Osteocalcin
OVX-F: Ovariectomized-fractured
SOD: Superoxide dismutase
ROS: Reactive oxygen species
TGF-β: Transforming growth factor beta
TNF-α: Tumor necrosis factor -alpha

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Conceptualization: Florence NgueguimTsofack, ThéophileDimo, Pierre Kamtchouing; Methodology: AngèleBidja, Jean Philippe DjientcheuTientcheu, Olivier NdogoEteme; Formal analysis and investigation: AngèleBidja, Jean Philippe DjientcheuTientcheu, RodrigueFifen, Paul Desire DjomeniDzeufiet, Writing – original draft preparation: AngèleBidja, Jean Hubert Donfack; Olivier NdogoEteme, Jean Philippe DjientcheuTientcheu; Writing – review and editing: Jean Hubert Donfack,RacelineKamkumoGounoue, Florence NgueguimTsofack; Supervision: Florence NgueguimTsofack, Kamtchouingpierre and ThéophileDimo. All authors read and approved its final version.

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