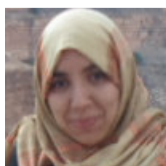


**TOXICITY AND ANTI-INFLAMMATORY EFFECTS OF METHANOLIC EXTRACT OF *UMBILICUS RUPESTRIS* L. LEAVES (CRASSULACEAE)****AFAFBENHOUDA* AND MOULOUD YAHIA***Biotechnology's Laboratory of the Bioactive Molecules and the Cellular Physiopathology, Department of Biology, University of BATNA, ALGERIA***ABSTRACT**

Umbilicus rupestris (UR) is a medicinal plant used in traditional medicine against inflammation and irritation of the skin. The aim of this work is to study *in vivo* on Wistar rats the acute and subacute toxicity of the methanolic extract of *U. rupestris* (URMeOH), anti-inflammatory effects by using several methods: The inflammation induced by carrageenan, the inflammation induced by the inflammation mediators, arthritis, peritonitis in Wistar rats and finally, effect of URMeOH on the stabilization of erythrocyte membrane *in vitro*. In the acute toxicity study, the LD50 of URMeOH was found higher than 2000 mg / kg b.w; and this dose did not cause neither death nor signs of toxic manifestations on treated rats. In the subacute toxicity study, no toxic signs were observed after 28 days of treatment with therapeutic doses 100 and 200 mg / kg b.w. The inflammation induced by carrageenan, chemical mediators (histamine and serotonin) and formalin solution was significantly inhibited and dose-dependent manner compared to the negative control after the administration of URMeOH (100 mg and 200 mg / kg B.w.). Also, the URMeOH revealed that it has an effect on the stabilisation of erythrocyte membrane after induction of hemolysis and it has the anti-peritonitis activity by inhibiting leukocytes migration. This study demonstrated the anti-inflammatory effects of URMeOH and that confirmed its use in traditional medicine.

KEYWORDS: *Umbilicus rupestris*, methanolic extract, toxicity, paw edema, leukocytes migration; anti-inflammatory activity.

**A. BENHOUDA***Biotechnology's Laboratory of the Bioactive Molecules and the Cellular Physiopathology, Department of Biology, University of BATNA, ALGERIA*

*Corresponding author

INTRODUCTION

The inflammation is caused by the release of chemical mediators from tissues and migratory cells. The most involved mediators are: prostaglandins (PGs), leukotrienes (LTs), histamine, bradykinin and recently PAF and interleukine-1 (LTs) ¹. In the presence of the inflammatory agent, the cellular membranes induce the activation of phospholipase A2 followed by the release of arachidonic acid and inflammatory mediators such as: the cytokines, serotonin, histamine, leukotrienes and prostaglandins which increase the vascular permeability, and facilitate the migration of leukocytes to the site of inflammation ^{2,3,4}. Currently, the pharmacologists are very interested to look through the word the most powerful anti-inflammatory products and more secure ⁵. The natural products play an important role in human health in the prevention and the treatment of inflammatory process ⁶. *Umbilicus rupestris* (Salisb.) Dandy is a perennial plant, belongs to the family of *Crassulaceae*. It presents on the rocks, the old walls and in mountains; only in North Africa. Leaves of this plant are used in traditional medicine against the ignitions of skin, wounds, burns, disinfectant, parasitocidal. Infused (leaves) of this plant is used like an ophthalmic disinfectant ⁷. Our work is to evaluate the acute and the subacute toxicity of this plant and also the anti-inflammatory activity on Wistar rats.

MATERIALS AND METHODS

Chemical products

Methanol (Sigma-Aldrich), Formaldehyde CH₂O (EDEN LABO), Carrageenan (Sigma-Aldrich), Indomethacin (Sigma-Aldrich), histamine (Sigma-Aldrich), Serotonin (Sigma-Aldrich) are used in this work.

Collection of the plant

The leaves of *Umbilicus rupestris* were harvested in March 2012 from the Wilaya of Batna region Tibhirine. Botanical species identification was realised by Dr. B. OUDJHIH, Botanical Laboratory, Department of agronomy, University of EL-Hadj Lakhdar - Batna. The leaves of this plant freshly harvested were washed and then dried in the

shade for 40 days in a dry and aerial place for the extraction of active compounds. They were then coarsely ground and collected in clean bags.

Extraction by organic solvents

1 Kg of powdered leaves was extracted with 5 L of petroleum ether for three times. Then, the marc was dried and extracted with 5 L of chloroform for three times and with 5 L of methanol for three times and the supernatants were filtered separately using cotton and Whatman filter paper. The solvents were then evaporated under reduced pressure (204 mbar) and controlled temperature (30 °C) using a vacuum rotary evaporator (Buchi Rotavapor).

Phytochemical screening

The phytochemical screening of URMeOH was realised by using the method of ⁸. Phytochemical constituents such as: phenolic compounds, terpenoids, saponins, alkaloids, steroids and tannins were analysed qualitatively.

Animals

Wistar rats weighted (140-170 g) provided by the Pasteur Institute – Algiers. These rats were allowed a favorable conditions before and during the experiment: Temperature (23 ± 2) ° C, relative humidity 50 -55 % with 12 hours light / 12 hours night cycle respectively. The food and water were given *ad libitum*.

The acute toxicity study

The acute toxicity of the methanolic extract of *U. rupestris* was evaluated on female Wistar rats weighted (150-170 g) according to OECD guideline 420 (OECD, 2001) which limits the dose at 2000 mg / kg b.w. ⁹. Female rats (140-160 g) were divided in groups. Four groups of six rats received URMeOH by gavage using the concentrations (500, 1000, 1500 and 2000 mg/Kg b.w) respectively, with a volume of 10 ml / kg b.w., the control group received the distilled water (10 ml / kg b.w). The observations were followed 30 min, 1, 2 and 4 hours each day for 14 days. The changement of the skin, morbidity, respiratory movements, the number of dead animals

were recorded for each group. LD50 was calculated according to the arithmic method of Karbar.

The subacute toxicity study

Both doses of 100, 200 mg / kg b.w. were selected for subacute toxicity test. Three groups were used and each group contained six rats (140 – 160 g) (3 males + 3 females). Group I considered as a negative control and received distilled water with a volume of 10 ml / kg b. w. Group II and III received URMeOH with 100 and 200 mg / kg b.w. / day respectively with the volume of (10 ml / kg b.w. in distilled water) every day for 28 days. The body weight and mortality were recorded during this period, which were considered as toxic manifestations. After 28 days, these animals were fasted all the night and dissected after anesthesia with chloroform, biochemical analyses were realised for the blood samples which were taken from ocular sinus. The internal organs as: liver, kidneys, lungs and heart were removed, weighted and analysed for the lesions .Finally they conserved in formalin 10% for the histopathological study ¹⁰.

Biochemical analyses

Blood is collected in heparin tubes and centrifuged at 3500 rpm for 5 min. After centrifugation, the biochemical analyses were effected of some parameters like :Aspartase aminotransferase (ASAT) or SGOT , alanine aminotransferase (ALAT) or SGPT, alkaline phosphatase (ALP) , cholesterol,

triglycerides , urea, glucose , creatinine, Bilirubin (Bil).

Histopathological study

The histopathological study was done according to the method of ¹¹. Organs (liver and Kidney) were fixed in a formalin solution 10%, after these organs are embedded in the paraffin, these tissues were cut with microtome 5 µm of thickness and are mounted on slides and stained with the hematoxylin-eosin.

Anti-Inflammatory Activity

Inflammation induced by Carrageenan

The anti-inflammatory test was effected according to the protocol of ¹². Four groups of rats Wistar, each one received the following treatments : Group I: received URMeOH (100 mg / kg b.w) , Group II : received URMeOH (200 mg / kg b.w) , Group III received distilled water as the negative control , Group IV received indomethacin (10 mg/Kg b.w.) as reference product. The paw volume of the rats was measured by vernier caliper before the treatment (Vol 0) . One hour after treatment, 0.1 ml of carrageenan 5 % prepared in NaCl 0.9% was administered by the subcutaneous route in the right hind paw. Then the volume of the paw was taken at 0.5 , 1 , 2, 3 , 4, 5 , and 6 hours after the administration of carrageenan (Volt) . The oedema was expressed as an increase in paw volume (ΔVol) , and the percentage of inhibition (I %) for each treatment was obtained as follows :

$$\Delta\text{Vol} = \text{Volt} - \text{Vol}_0$$

$$\text{where } I (\%) = [(\Delta\text{Vol}_c - \Delta\text{Vol}_{tr}) / \Delta\text{Vol}_c] \times 100 .$$

$$\Delta\text{Vol}_{tr} = \text{increased volume of right paw in the treated group}$$

$$\Delta\text{Vol}_c = \text{increased volume of right paw in the control group}$$

Induction of Inflammation by Mediators

In inflammation induced by mediators, Group I: received distilled water as negative control, group II: received the indomethacin (10 mg / kg b.w) as a reference product, group III and group IV: received URMeOH (100 and 200 mg/Kg b.w. respectively) . 1 hour after the oral administration of treatments, oedema was induced by subcutaneous injection of 0.1 ml of a freshly prepared solution of serotonin (10 mg / mL) and histamine (1 mg / ml) in right paw of rats. The paw volume was measured after 1 hour of injection of histamine and serotonin ¹³.

The percentage of inhibition induced by each drug was calculated as follow

where $I (\%) = [(\Delta\text{Vol } c - \Delta\text{vol } tr) / \Delta\text{Vol } c] \times 100$.

$\Delta\text{Vol } tr$ = increased right paw volume in the treated group

$\Delta\text{Vol } c$ = increased volume of the right paw in the control group.

Inflammation Induced by Formalin Solution

The activity was affected according to the method of ¹⁴. Four groups were received the following treatments: Group I: a positive control was treated with indomethacin (10 mg / kg b.w), Group II and III were received the URMeOH (100 and 200 mg / kg b.w.respectively). 30 min later, 0.1 ml of formalin 2 % prepared in NaCl 0.9% was injected subcutaneously in the right hind paw of rats . The paw volume was calculated using the vernier caliper before administration of the treatment and after 1, 2 , and 4 hours after injection of the formalin solution. These same animals were subjected to a chronic inflammation. They were treated with URMeOH every day during 10 successive days, knowing that the second injection of the

formalin solution has been given in the third day, the volume of the paw was taken every day during these 10 days.

Peritonitis induced by Carrageenan

The activity was affected according to the method of ¹⁵ with modifications. 0, 5 ml of solution of Carrageenan 1% prepared in a saline solution was injected intraperitoneally 1 hour after administration of the following treatments: Group I received indomethacin (10 mg / kg b.w), Group II and III were administered URMeOH (100 and 200 mg / kg b.w.) respectively , and group IV received distilled water and used as a negative control . After 4 hours, the animals were dissected. A saline solution containing EDTA (1 mM) was used to collect the peritoneal fluid .

The counting of leukocytes and neutrophils are effected, leukocyte inhibition was calculated as follow:

$$I\% = (1 - N_t / N_c) \times 100$$

N_t : Number of leukocytes in the treated groups

N_c : Number of leukocytes in the control group.

The change of neutrophils was calculated by the following equation:

$$\text{Change neutrophils} = [N_{neu_t} - N_{neu_c} / N_{neu_c}] \times 100$$

N_{neu_t} : represents the number of leukocytes in the treated groups.

N_{neu_c} : represents the number of leukocytes in the control group.

Effects of URMeOH on plasmic membrane

The method described by ¹⁶, 5 ml of human blood was collected in EDTA tubes. After centrifugation at 3000 rpm for 5 min, this blood will be washed with the same volume of saline solution. The volume of the blood was measured and reconstituted as a

suspension of 40 % with an isotonic buffer solution (pH = 7.4) . (The composition of the buffer solution (g / l) : NaCl (4.4 g) , NaH_2PO_4 (1.6 g) and Na_2HPO_4 (7.6 g)) . **Hemolysis Induced by Heat** Four series of tubes were realized, serie I : consist four tubes of 5 ml containing concentrations 200

µg / ml of URMeOH in isotonic buffer solution . Serie II: consist four tubes of 5 ml containing concentrations containing 400 µg / ml of URMeOH in isotonic buffer solution. Serie III: consist four tubes of 5 ml containing concentrations 200 µg / ml of indomethacin in isotonic buffer solution as reference. Serie IV: consist four tubes of 5 ml containing isotonic buffer solution. 0,1 ml of erythrocyte suspension was added to all these tubes of

the four series. We took a first pair of tubes among different series and were incubated in water bath 54°C for 20 min. The other pair of tubes were put in a freezer -10°C for 20 min. After all these manipulations, all tubes were centrifuged at 1000 rpm for 5min and the absorbance was taken by using a spectrophotometer (λ =540 nm).

The percentage of hemolysis was calculated as follow

$$\text{Inhibition of hemolysis (\%)} = [1 - (At_2 - At_1 / Ac - At_1)] \times 100$$

At₁ = absorbance of unheated test

At₂ = absorbance of the heated test

Ac = absorbance of heated control

Hemolysis induced by hypotonicity

Four series of tubes were realized, serie I: consist four tubes of 5 ml containing concentrations 200 µg / ml of URMeOH (two tubes were prepared in isotonic buffer solution and other tubes were prepared in hypotonic solution (distilled water)). Serie II: consists four tubes of 5 ml containing concentrations 400 µg / ml of URMeOH (two tubes were prepared in isotonic buffer solution and other tubes were prepared in hypotonic solution) . Serie III: consists four tubes of 5 ml containing concentrations 200 µg / ml of indomethacin (two tubes were

prepared in isotonic buffer solution and other tubes were prepared in hypotonic solution). Serie IV: consists four tubes of 5 ml (two tubes containing isotonic buffer solution and the other tubes containing distilled water) as a negative control . 0,1 ml of erythrocyte suspension was added to all these tubes of the four series, the mixture was gently stirred and subjected to incubation for 1 h at room temperature (25°C) . At the end of incubation, the tubes were centrifuged at 3000 rpm for 5 min and the absorbance of the supernatant measured at λ =540 nm).

The percentage of inhibition of hemolysis is calculated by

$$\text{Inhibition of hemolysis (\%)} = [1 - (Ath - At_i / Ach - At_i)] \times 100$$

At_i = absorbance of the test in isotonic solution

Ath = absorbance of the sample in a hypotonic solution

Ach = absorbance of the control sample in a hypotonic solution

RESULTS

Phytochemical screening

Preliminary phytochemical study of URMeOH extract is presented in (Table 1). Phytochemical analysis of this extract

showed the presence of compounds which are known with their anti- inflammatory activities: Saponins , flavonoids , steroids , terpenoids and tannins

Table 1
Phytochemical screening of URMeOH.

Sample	Phytochemical constituent	Result
URMeOH	Alkaloids	+
	Saponins	+++
	Flavonoids	++
	Tannins and polyphenolic compounds	++
	Terpenoids	+++
	Steroids	++

+++ : very intense reaction, ++ : intense reaction, + : positive reaction

Test of toxicity

The acute toxicity study

The results of this study are presented in (Table 2). In this study, the oral administration of URMeOH with all doses (til 2000 mg kg⁻¹) induced neither visible sign of acute toxicity nor death in tested rats during the period of observation, therefore the LD50 ≥ 2000 mg / kg b.w .

Table 2
Determination of LD50 by arithmetic method of Karbar

Groups	Number of rats	Number of dead animals	Dose difference (a)	Mean mortality (b)	Probit (a×b)
Control group	6	0	No death	Not calculated
<i>U. rupestris</i> 100 mg /kg	6	0	100	No death	Not calculated
<i>U. rupestris</i> 200 mg /kg	6	0	100	No death	Not calculated
<i>U. rupestris</i> 500 mg /kg	6	0	300	No death	Not calculated
<i>U. rupestris</i> 1000 mg /kg	6	0	500	No death	Not calculated
<i>U. rupestris</i> 2000 mg /kg	6	0	1000	No death	Not calculated

The subacute toxicity study

Regarding to the subacute toxicity, no deaths were recorded during the period of treatment with therapeutic doses in both groups (control and treatment groups) during 28 days. The animals did not show changes in general behavior and other physiological activities.

The results showed that there was not significant difference ($p > 0.05$) in body weight and organ weights between control and treated animals after 28 days of treatment (Table 3).

Table 3
Effect of URMeOH on body and organ weights (g) of rats in subacute toxicity.

	Control (2ml water/100g b.w)	URMeOH (100 mg/Kg b.w)	URMeOH (200 mg/Kg b.w)
Body weight			
Initial	174.0 ± 3.0	173.7 ± 1.52	174.3 ± 1.52
Final	241.3 ± 2.51	244.0 ± 3.05	241.7 ± 1.52
Organ weight			
Liver	11.65 ± 0.01	11.54 ± 0.03	11.57 ± 0.07
Kidney	1.21 ± 0.01	1.26 ± 0.02	1.28 ± 0.03
Lungs	1.08 ± 0.01	1.11 ± 0.00	1.11 ± 0.030
Heart	0.92 ± 0.03	0.90 ± 0.02	0.88 ± 0.03

Data are expressed as mean ± s.d. (n=6). No statistical difference between control and URMeOH groups ($p > 0.05$)

Biochemical estimations

The results of biochemical analyses after administration of URMeOH showed that there were not significant increasing ($P \geq 0.05$) in different parameters : glucose (g / L), urea (g / l), creatinine (mg / L) Cholesterol (g / L), triglyceride (g / l), ALP, SGOT, SGPT and Bilirubin in different groups of animals treated with URMeOH (100 and 200 mg / kg b.w.) in comparison to the negative control (Table 4).

Table 4
Effect of methanolic extract from *U.rupestris*' leaves on biochemical parameters of rats in subacute toxicity (mean \pm SEM, n= 6)

Parameters	control group	Groups treated with URMeOH	
		100 mg/kg b.w	200 mg/kg b.w
Glucose (g/L)	0.92 \pm 0.05	1.04 \pm 0.11	1.10 \pm 0.05
Urea (g/L)	0.44 \pm 0.06	0.40 \pm 0.08	0.42 \pm 0.05
Creatinine (mg/L)	12.75 \pm 0.90	11.70 \pm 2.05	11.98 \pm 0.54
Cholesterol (g/L)	0.82 \pm 0.04	0.77 \pm 0.02	0.81 \pm 0.03
Triglycerides (g/L)	0.63 \pm 0.03	0.68 \pm 0.03	0.61 \pm 0.02
SGOT (U/L)	131.3 \pm 1.56	136.9 \pm 3.94	139.5 \pm 4.60
SGPT (U/L)	46.37 \pm 2.90	43.15 \pm 1.65	46.30 \pm 2.52
ALP (U/L)	149.7 \pm 1.54	153.1 \pm 1.42	151.5 \pm 2.33
Bilirubin (mg/L)	0.81 \pm 0.01	0.84 \pm 0.05	0.81 \pm 0.02

Values expressed as mean \pm STD; Significance with Tukey's test following one way ANOVA is evaluated as $p > 0.05$

No significant difference. SGOT –Serum Glutamate oxaloacetate transaminase; SGPT – Serum Glutamate pyruvate transaminase, ALP- Alkaline phosphatase.

Hematological estimations

The hematological estimation has shown that there were not any changes in hematological parameters ($p > 0.05$) in hemoglobin (Hb), red blood cells (RBC), white blood cells (WBC), hematocrit (Ht) and platelets (PLT) in all treated groups compared to the control group (Table 5).

Table 5
Effect of URMeOH on hematological parameters of rats in subacute toxicity, n= 6 (3 males +3 females).

Parameters	Control group	<i>U.rupestris</i> treated group	
		100 mg/kg b.w	200 mg/kg b.w
RBC ($\times 10^6$ / μ L)	7.75 \pm 0.01	7.70 \pm 0.02	7.72 \pm 0.04
Leukocyte ($\times 10^6$ /mL)	8144.78 \pm 5.03	8152.87 \pm 6.11	8147.45 \pm 2.00
Hb (g/dL)	14.91 \pm 0.06	14.92 \pm 0.04	15.02 \pm 0.11
Ht (%)	44.58 \pm 1.03	42.88 \pm 0.72	44.64 \pm 0.51
PLT ($\times 10^3$ / μ L)	794.3 \pm 3.03	803.3 \pm 5.03	795.0 \pm 4.35
MCV (fL)	56.07 \pm 1.19	55.58 \pm 2.11	59.57 \pm 1.22
MCHC (g/dL)	36.81 \pm 2.17	34.71 \pm 0.94	35.33 \pm 0.39
WBC ($\times 10^3$ / μ L)	7.33 \pm 0.11	7.78 \pm 0.22	7.62 \pm 0.33
Neutrophils (%)	25.58 \pm 1.56	26.77 \pm 0.90	24.36 \pm 0.61
Eosinophils (%)	1.68 \pm 0.02	1.73 \pm 0.03	1.56 \pm 0.11
Basophils (%)	00.00 \pm 0.00	00.00 \pm 0.00	00.00 \pm 0.00
Lymphocyte (%)	65.33 \pm 1.04	65.17 \pm 1.61	65.48 \pm 1.54
Monocyte (%)	1.58 \pm 0.02	1.62 \pm 0.03	1.63 \pm 0.05

Values expressed as mean \pm STD; Significance with Tukey's test following one way ANOVA is evaluated as: No statistical difference between control and URMeOH groups ($p > 0.05$).

MCV – mean corpuscular volume; MCHC – mean corpuscular hemoglobin concentration, WBC - white blood cells

Histopathological investigation

After histopathological study, it was found that there were no changes after the URMeOH administration with 100 mg / kg and 200 mg / kg b.w.doses in rats after a period of 28 days compared with negative control (Figure 1 and 2).

Figure 1

Photomicrograph of Liver histology of treated and untreated albino rats with methanolic extract of *Umbilicus rupestris*'s leaves [A]control group, [B] URMeOH [100 mg/kg b.w], [C] URMeOH[200 mg/kg b.w]. Liver sections stained with hematoxylin and eosin (90 X).

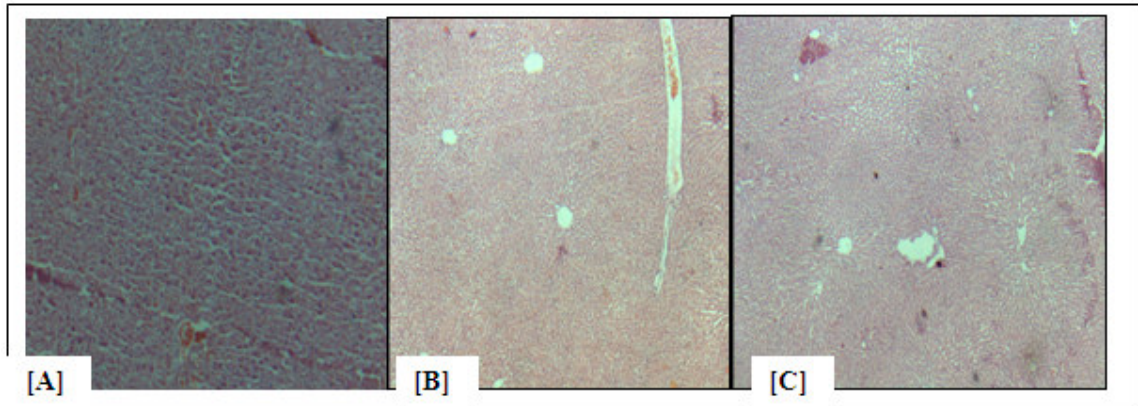
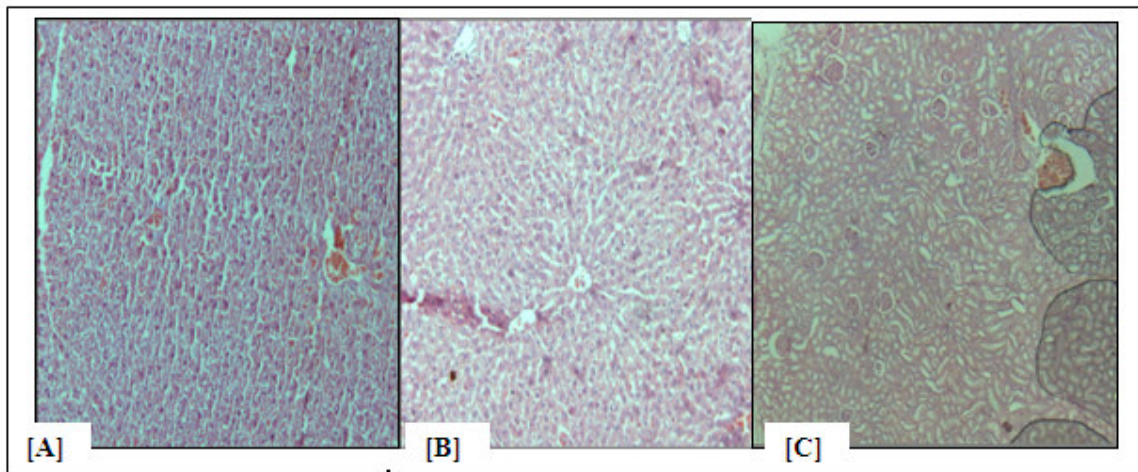


Figure 2

Photomicrograph of Kidney histology of treated and untreated albino rats with methanolic extract of *Umbilicus rupestris*'s leaves.[A]control group, [B] URMeOH [100 mg/kg b.w], [C] URMeOH [200 mg/kg b.w].Kidney sections stained with hematoxylin and eosin (90X).



Anti-inflammatory activity

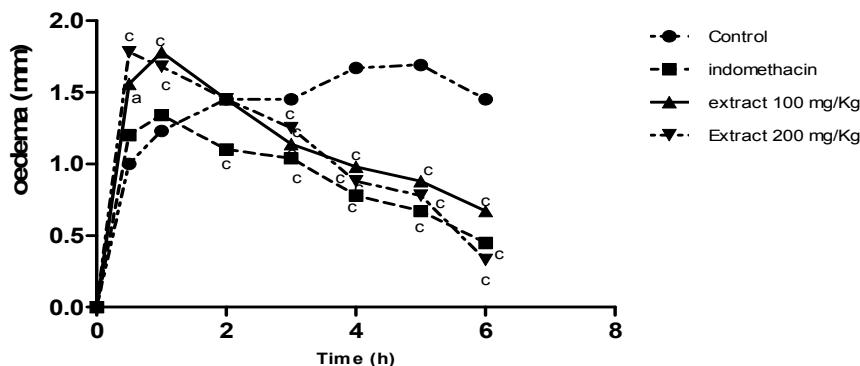
Inflammation induced by Carrageenan

As illustrated in Graph 1, the subcutaneous injection of the carrageenan solution produced an inflammatory oedema which increased until the fifth hour after injection. The methanolic extract (100 and 200 mg / kg b.w) induced

significantly ($P < 0.001$) an anti-inflammatory effect which gradually increased and reached the maximum (54.64 % and 74.07 %) respectively . Indomethacin (10 mg / kg b.w) showed an anti-inflammatory effect of 67.04 % ($P < 0.001$) in the sixth hour.

Graph 1

Anti-inflammatory effect of URMeOH on rat hind paw oedema induced by carrageenan.



Each point represents the mean \pm SEM of 6 animals; ^aP < 0.05; ^bP < 0.001, ^cP < 0.0001 statistically significant compared to their respective control.

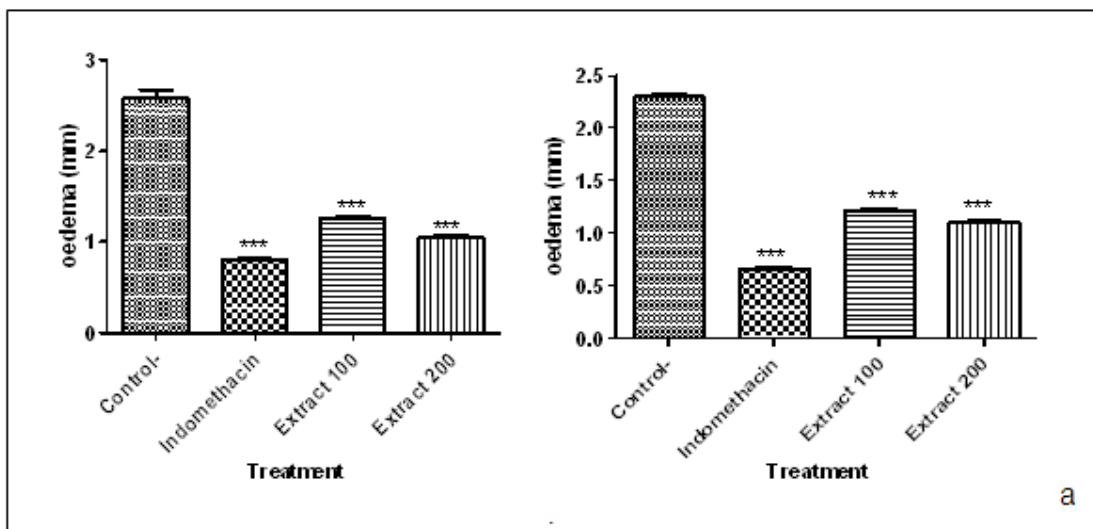
Effect of the extract on inflammatory mediators

Graph 2 showed that the effects of URMeOH on paw edema induced by serotonin and histamine. The dose 200 mg / kg b.w of URMOH showed a significant anti-inflammatory activity and dose-dependent with a maximum effect of 59.47 % in the

eodema induced by histamine and an effect of 51.80 % in edema induced by serotonin. Indomethacin prevented significantly (P < 0.05) the inflammation induced by histamine and serotonin with 68.91 % and 71.12 % respectively.

Graph 2

Effects of URMeOH on paw oedema induced by histamine (a) and serotonin (b).

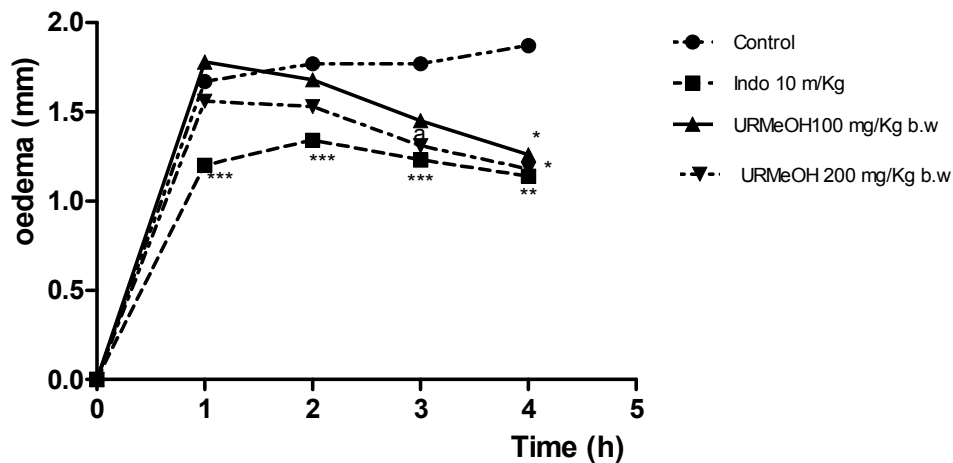


Each bar represents the mean \pm SEM of 6 animals; ^cP < 0.0001 statistically significant compared to their respective control.

Oedema induced by formalin solution acute

URMeOH prevents significantly and dose-dependent manner the acute inflammation induced by the formalin. The dose 200 mg / kg b.w. reduced significantly the formation of eodema of the paw with 25.19 % and 36.33 % in the 2nd and 4th hour respectively. Indomethacin inhibited significantly acute inflammation induced by the formalin with an effect of 39.42 % at fourth hours (Graph 3).

Graph 3
Effects of URMeOH on acute inflammation of paw oedema induced by formalin.



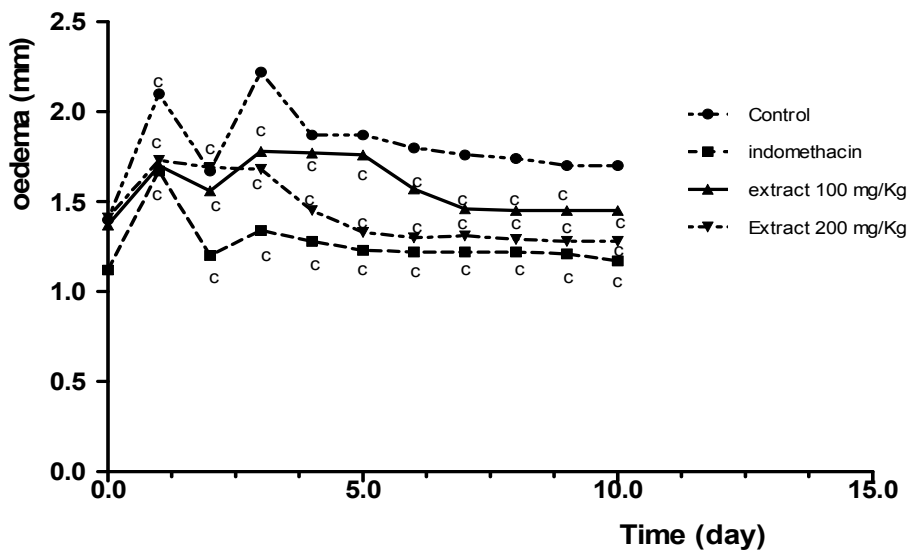
Each point represents the mean \pm SEM of 6 animals; ^aP < 0.05, ^bP < 0.001; ^cP < 0.0001 statistically significant compared to their respective control.

Chronic

The extract showed a significant inhibition of chronic inflammation. The extract (100 and 200 mg / kg b.w.) prevented significantly the arthritis induced by formaldehyde 19.07%

17.80% respectively in the 3rd day. At the same doses, URMeOH prevented a chronic inflammation with 15.21% and 22.33% respectively in the 10th day (Graph 4).

Graph 4
Effects of URMeOH on chronic inflammation of paw oedema induced by formalin.



Each point represents the mean \pm SEM of 6 animals; ^aP < 0.05; ^bP < 0.001, ^cP < 0.0001 statistically significant compared to negative control.

Peritonitis induced by Carrageenan

The URMeOH also prevented peritoneal leukocyte migration (41.63 % and 61.60 % at doses of 100 and 200 mg / kg b.w. respectively) while the inhibition produced by indomethacin (10 mg / kg b.w.) was found 47.14 % (Table 6).

Table 6
Effect of URMeOH on Leukocytes Migration in peritonitis induced by Carrageenan on Wistar rats.

Treatment	Leukocytes (10^5 ml^{-1})	Leukocytes inhibition	Neutrophils (10^5 ml^{-1})	Neutrophils changes
Control –	5.26 ±0.64	-	2.84±0.05	-
URMeOH 100 mg/kg b.w	3.07±0.08	41.63	0.67±0.02	23.59
URMeOH 200 mg/kg b.w	2.02±0.09	61.59	1.21±0.03	42.60
Indomethacin 10 mg/kg b.w	2.78±0.09	47.14	0.86±0.04	30.28

Values are mean±S.E.M. (n=6). Experimental groups were compared with control $p \leq 0.05$.

Effect extract on stability of the membrane

URMeOH has an effect on the stability of red cells membrane by inhibiting the hypotonicity with effect of 34.12 % and 40.00 % for doses

200 and 400 $\mu\text{g} / \text{ml}$ respectively, and inhibiting hemolysis induced by heat with percentages of 22.05 % and 29.13 % for doses 200 and 400 $\mu\text{g} / \text{ml}$ respectively (Table7).

Table 7
Effects of URMeOH on membrane stabilization.

Treatment	Percentage inhibition of haemolysis induced by heat	Percentage inhibition of haemolysis hypotonicity induced
URMeOH 200 $\mu\text{g}/\text{mL}$	22.05	34.12
URMeOH 400 $\mu\text{g}/\text{mL}$	29.13	40.00
Indomethacin 100 $\mu\text{g}/\text{mL}$	51.66	53.28

DISCUSSION

The observations during the oral toxicity showed that *U.rupestris* is non toxic. For the subacute study, the higher dose level of 200 mg/Kg (1/10th of maximum tolerated dose) and low dose level are selected (1/20th of maximum tolerated dose)¹⁷. The Biochemical analyses showed that there is not any change in glucose and cholesterol level that involves that URMeOH has no effect on the metabolism of lipid and carbohydrate in rats. For a toxic liver, transaminases: ASAT and ALAT are considered as good indicators and biomarkers of liver function¹⁸. The hematopoietic system is very sensitive to toxic substances and very important in the physiology and pathology of the animal and the human being¹⁹. The inflammation induced by Carrageenan is a sophisticated method to detect active oral anti-inflammatory agents²⁰. The creation of the oedema is due to the release of pharmacological mediators, histamine, 5-HT, kinins, PGs and pro-inflammatory cytokines.

Carrageenan is a sulfated polysaccharide obtained from algae which is an agent widely used to induce inflammation and to increase vascular permeability²¹. The inflammation induced by carrageenan causes the generation of two phases²². The early phase (1 to 2 h) resulting from the release of histamine, serotonin and leukotrienes, while a delayed phase (more than 3 h) is due by the release of prostaglandins and neutrophils infiltration²³. The URMeOH has an anti-inflammatory effect during the first two hours (early phase) is due to the inhibition of the synthesis of histamine and serotonin, and an effect after the third hour by inhibiting the cyclooxygenase and following inhibition of prostaglandin synthesis²⁴. Histamine and serotonin are the effective vasodilator substances and are known by their effectiveness to increase the vascular permeability²⁵ and then the extract can be inhibit the synthesis of histamine and serotonin²⁶. The acute inflammation

induced by the formalin results damages to the cells causing the production of endogenous mediators such as: histamine, serotonin, prostaglandins and bradykinin. And as has been proven by²⁷ URMeOH may interfere with these mediators to prevent inflammation. The inflammation induced by formaldehyde is considered as a typical example in the acute and chronic inflammation in researching of antiproliferative and anti-arthritic substances²⁷. Leukocytes migrate to the site of inflammation in response to the chemotactic stimulus²⁸. Our results are almost confused with what was found by²⁹ that the reduction of the neutrophils during the peritonitis can be due to the production of the pro-inflammatory cytokines, like :TNF- and IL - 1 β . And thus the URMeOH extract reduces the migration of the neutrophils in the peritoneal cavity by its effect on these cytokines²⁹. TNF induces the chemotaxis of neutrophils and lymphocytes T and increases the expression of the adhesion molecules²⁹. According to³¹, URMeOH has an effect on the stability of the erythrocytes membrane against hypotonicity, this is due to the inhibition of the release of phospholipases which trigger the formation of inflammatory mediators).Lysosomes lysis during inflammation liberates enzymes (such as the

membrane of lysosomes are similar to those of the red cells) , URMeOH inhibites the membrane lysis of red cells induced by hypotonicity and heat, so, URMeOH has an anti -inflammatory effect on the lysis of lysosomes membrane³².The phytochemical screening ofURMeOH shows the presence of flavonoids , steroids, tannins and terpenoids which were played an important role in the inhibition of the enzymes involved in the synthesis of chemical mediators of inflammation and inhibition of metabolism of arachidonic acid^{33, 34}.

CONCLUSION

In conclusion, the results of this study support the use of the plant *U.rupestris* in treating inflammation in traditional medicine. The anti-inflammatory activity of URMeOH may be due to the inhibition of mediators of inflammation, prostaglandin synthesis, inhibition of leukocytes migration and further stabilization of erythrocyte membrane. The work must be research the exact mechanism responsible for the anti -inflammatory effect of the methanolic extract of *U . rupestris* and the identification of compounds that play the crucial role .

REFERENCES

1. Van J., and Botting R, Inflammation and the mechanism of action of antiinflammatory drugs . FASEB J., 1:89-96, (1987).
2. Dassoler M., Schwanz M., Busseto F., Moreira E.A., Gutierrez L, Perfil fitoquímico e ensaio farmacológico de *Averrhoa carambola* L. (Oxalidaceae). J. Bras. Fitom., 2, 4–8 (2004).
3. Falcão H., Lima IO. , Santos VL, Dantas HF , Diniz F M, Barbosa-Filho JM, Batista LM : Review of the plants with anti-inflammatory activity studied in Brazil, Braz. J. Pharmacognosy, 15 : 381–391,(2005).
4. De Cássia da Silveirae Sá R, Andrade LN and de Sousa D.P, Anti-Inflammatory Activity of Monoterpenes .Molecules, 18 :1227-1254,(2013).
5. Kumari S.T.K. , Lincy M.P., Muthukumarasamy S .and Mohan V .R, Anti- inflammatory activity of *sarcostemma secamone* bennet whole plant against carrageenan induced paw edema.Bioscience Discovery, 3(3) : 288-291,(2012).
6. Gupta R.K., Hussain T., Panigrahi G., Das A., Singh G.N., Sweetey K, Hepatoprotective effect of *Solanum xanthocarpum* fruit extract against CCl4 induced acute liver toxicity in extract against CCl4 induced acute liver toxicity in experimental animals. Asian Pac J Trop Med, 4: 964-968, (2012).
7. Benhouda A., Yahia M., Benhouda D., Bousnane N.E., Benbia S., Hannachi N.E., Ghecham A, Antimicrobial and Antioxidant activities of various extracts of *Hyoscyamus albus* L. and *Umbilicus*

- rupestris* L. leaves. Algerian J. Nat. Products, 2:14-17,(2014).
8. Harborne J.B, Phytochemical methods, A guide to modern techniques of plant analysis., *Thirdth edition. Vol 4, New York: Chapman and Hall Int.* 138-147,(1998).
 9. Lip N.R, Cotruvo R., Hill J., Bruce R., Stitzel K., Walker A., Chul Goddard M., Segal L., Springer J., Myers R.C, Comparison of the up and down, Conventional LD50 and fixed dose Acute toxicity procedures. *Fd Chem. Toxicology*, 33: 223-231,(1995).
 10. OECO."Organisation for Economic Co-Operation and Development (OECD), Guidelines for Testing of Chemicals, Repeated Dose 28-day Oral Toxicity Study in Rodents #407, Paris, France, (1995), Accessed on "12 Augs 2012 . [http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono\(2012\)22&doclanguage=en](http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2012)22&doclanguage=en)
 11. Lamb GM. Manual of veterinary techniques in Kenya. 2nd edition. Vol 2 *Kenya, Ciba-Geigy*, 100-104,(1981).
 12. Winter C.A., Riseley E.A. and Nuss G.W, Carrageenan-induced Oedema in the Hind Paw of the rats as an Assay for Anti-inflammatory Drugs. *Experimental Biology and Medicine*, 111:544-547,(1962).
 13. Singh S., Majumdar D.K., and Rehan H.M.S ,Evaluation of anti-inflammatory potential of fixed oil of *Ocimum sanctum* (Holybasil) and its possible mechanism of action. *Journal of Ethnopharmacology*, 54 (1) 19–26,(1996).
 14. Dimo T., Fotio A., Nguelefack T., Asongalem E., and Kamtchouing P, Antiinflammatory activity of leaf extracts of *Kalanchoe crenata* Andr. *Indian Journal of Pharmacology*, 38 (2) : 115–119,(2006).
 15. Bastos L.F., Merlo L.A., Rocha L.T., Coelho M.M, Characterization of the antinociceptive and anti-inflammatory activities of doxycycline and minocycline in different experimental models. *Eur J Pharmacol*, 576: 171-179,(2007).
 16. Shinde U.A., Phadke A.S., Nair A.M., Mungantiwar A.A., Dikshit V.J., Sarsf M.N, Membrane stabilization activity- a possible mechanism of action for the anti-inflammatory activity of *Cedrus deodara* wood oil. *Fitoterapia*, 70:251–257,(1989).
 17. Hussain T., Fareed S., Siddiqui H.H., Vijaykumar M. , Rao C. V, Acute and subacute oral toxicity evaluation of *Tephrosia purpurea* extract in rodents . *Asian Pacific Journal of Tropical Disease*, 2(2): 129-132 ,(2012).
 18. Hilaly J.E., Israili Z.H. and Lyoussi B, Acute and chronic toxicological studies of *Ajuga iva* in experimental animals. *Journal of Ethnopharmacology*, 91(1):43–50 (2004).
 19. Adeneye A.A., Ajagbonna O.P., Adeleke T.I., Bello S.O, Preliminary toxicity and phytochemical studies of the stem bark aqueous extract of *Musanga cecropioides* in rats. *Ethnopharmacology*, 105: 374-379,(2006).
 20. Jaijoy K., Soonthornchareonnon N., Panthong A., Sireeratawong S, Anti-inflammatory and analgesic activities of the water extract from the fruit of *Phyllanthus emblica* Linn. *International Journal of Applied Research in Natural Products*, 3: 2, 28-35,(2010).
 21. Hammer K.A., Carson C.F., Riley T.V, Antimicrobial activity of essential oils and other plant extracts . *Journal of Applied Microbiology*, 86: 985–990,(1999).
 22. Tjolsen A., Berge O. G., Hunskaar S., Rosland J.H., and Hole K, The formalin test: an evaluation of the method. *Pain*, 51: 1, 5–17, (1992).
 23. Vinegar R., Schreiber W., and Hugo R, Biphasic development of carrageenin edema in rats. *The Journal of Pharmacology and Experimental Therapeutics*, 166: 96–103,(1969).
 24. Hirasawa N., Watanabe M., Mue S., Tsurufuji S. and Ohuchi K, Downward regulation of neutrophil infiltration by endogenous histamine without affecting vascular permeability responses in air-pouch-type carrageenan inflammation in rats. *Inflammation*, 15: 117-126,(1991).
 25. Skidmore I., Whitehouse M, Biochemical properties of anti-inflammatory drugs X: the inhibition of serotonin formation *in vitro* and inhibition

- of the esterase activity of α -chymotrypsin. *Biochem. Pharmacology*, 16:737-751,(1967).
26. Sowemimoa A., Onakoyaa M. , Fageyinbob M.S., Fadojua T, Studies on the anti-inflammatory and anti-nociceptive properties of *Blepharis maderaspatensis* leaves. *Rev Bras Farmacogn*, 23: 830-835,(2013).
 27. Banerjee S., Sur T.K., Mandal S., Das P.C., and Sikdar S, Assessment of the anti-inflammatory effects of *Swertia chirata* in acute and chronic experimental models in male albino rats. *Indian Journal of Pharmacology*, 32 (1): 21–24, (2000).
 28. Wagner J.G., Roth A.R, Neutrophil migration mechanisms, with an emphasis on the pulmonary vasculature. *Pharmacology Reviews Online*, 52: 349–374, (2000).
 29. .Alves C.F., Alves V.B.F., de Assis I. P., Clemente-Napimoga J. T., Uber-Bucek E., Dal-Secco D., Cunha F.Q., Rehder V.L.G., Napimoga M.H, Anti-inflammatory activity and possible mechanism of extract from *Mikania laevigata* in carrageenan-induced peritonitis. *Journal of Pharmacy and Pharmacology*, 61: 1097-1104,(2009).
 30. .Aitadafoun M., Mounieri C., Heymann S.F., Binistic C. , Bon C. and Godhold J, 4 Alkoxybenzamides as new potent phospholipase A2, inhibitors. *Biochemical pharmacology*, 51:737-742, (1996).
 31. Mounnissamy V.M., Kavimani S., Balu V., Drlin Q.S, Evaluation of anti-inflammatory and membrane stabilizing properties of ethanol extract of *Canjera rehedi*. *Iranian J Pharmacol Therapeut*, 6: 235–237,(2008).
 32. Oweyele B., Oloriegbe Y.Y., Balaogun E.A., Soladoye A.O, Analgesis and anti-inflammatory properties of *Nelsonia Canescens* leaf extract. *J Ethnopharmacol*, 99:153–156, (2005).
 33. Anosike C.A., Abonyi O., Ubaka C.M, Does the African garden egg offer protection against experimentally induced ulcers? *Asian Pac J Trop Med*, 4(2):163–164,(2011).

