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PHYTOTHERAPY AS NEW APPROACH TO TREAT SCORPION ENVENOMATION: EXPERIMENTAL STUDY

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ABSTRACT: *Citrullus colocynthis* is a wild common plant in arid and semi arid areas of Algeria which are characterized by high incidence of scorpion envenomation. It is used in Algeria to treat many diseases. In order to optimize and develop new therapeutic strategies for scorpion envenoming, a phytotherapeutic approach has been investigated. Pharmacological properties of *Citrullus colocynthis* were evaluated as new approach based on their local and systemic beneficial effects after scorpion envenomation mainly *Androctonus australis hector* venom *(Aah).* Local action (paw edema) and systemic effects (inflammatory, metabolic parameters, oxidative stress and hyperglycemia) were studied in pretreated mice with *Citrullus colocynthis* (50 mg/kg), 30 min before injection of sublethal dose of Aah venom (10 µg/20 g). Obtained results showed that injected *Citrullus colocynthis* before envenomation is able to protect animals against the toxicity of the venom. It significantly reduced paw edema, cell migration, exudation, hyperglycemia, and MDA. *Citrullus colocynthis* decreased also some inflammatory markers (MPO and EPO activities, CRP and C3) and maintain the level of CPK, ASAT and ALAT. *Citrullus colocynthis* appears to be a potential tool that can reduce pathophysiological effects induced after envenomation (inflammation and oxidative stress). According to these properties, it could be proposed as a symptomatic treatment of scorpion envenomation.

INTRODUCTION: In Maghreb region, especially in Algeria, immunotherapy is the most commonly used to treat stung patients by scorpion stings. This therapy is only effective when it is administered earlier after envenoming. The effectiveness of immunotherapy is limited by its use urgently after sting and thus has limitations that are inherent in the rapid response.

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Furthermore, most stung patients arrive at the hospital too late after the accident to take immune sera. The most incriminated scorpion species is Androctonus in the cited areas where Citrullus colocynthis is widely distributed. It is found in North Africa, the Sahara, Egypt, Saudi Arabia, India and Mediterranean region (Southern European) [1]. Citrullus colocynthis was known to have anti-inflammatory [2], antihistaminic [3], antioxidant [4, 5], anti-ulcer [6], antimicrobial [2], antihyperlipidemic [7] and antidiabetic [8, 9] effects. *Citrullus colocynthis* presents also therapeutical activity against a wide range of diseases, including inflammatory diseases, arthritis, gout [10], diabetes, high blood pressure [11, 12, 13], hemorrhoids [14, 15],

breast cancer [16], cardiovascular disorders [17] and liver disease [18]. There is obviously much still unknown about plants to treat scorpion envenomation. Scientific validation of usefulness of various species could form the basis for their use as alternative treatments [19].

The present investigation was undertaken to establish a new phytotherapeutic approach. The use of Citrullus colocynthis as symptomatic treatment for scorpion envenomation was based on its beneficial properties against pathophysiological effects induced after envenomation.

MATERIALS AND METHODS:

Venom: *Androctonus australis* Hector (Aah) venom was extracted, lyophilized and kept at 4°C until use.

Animals: NMRI male mice (20 ± 2 g; 02 months old) are provided from the central animal facility of Faculty of Biological Sciences (USTHB). Animals were housed in controlled temperature and humidity rooms, and received food and water before use. Experiments were carried out according to the European Community rules of the ethical Committee for animals' welfare.

Plant: Our study focused on the fruits of Citrullus colocynthis, which were harvested in February 2013 from Sidi Okba Willaya of Biskra (T = 13C°, pressure = 1021 hPa). The botanical species identification is made according to the classification proposed by Ozenda [20] and a voucher specimen deposited in the Faculty of Biology of USTHB.

Plant extracts preparation: Preparation of Citrullus colocynthis fresh fruit was carried out according to the method described by Marzouk *et al* (2010) [10].

Evaluation of the local action: In this study, 06 groups of 06 mice each have been used. Control group was injected with saline solution (NaCl 9‰) into the subplantar region of the right paw. Second group of mice was injected with a sublethal dose of Aah venom (10 µg/20 g body weight) into the subplantar region of the right paw. The third, fourth and fifth group received respectively pretreatment with Citrullus colocynthis 10 mg/kg, 25 mg/kg and 50 mg/kg by intraperitoneal route 30 min before envenomation. The level of paw edema in the right paw was evaluated 30 min, 2 and 5 hours after envenomation.

Evaluation of systemic action: The systemic action of Citrullus colocynthis was evaluated in envenomed mice with Aah venom (10 µg/20 g body weight by intraperitoneal route) with or without pretreatment with Citrullus colocynthis (50 mg/kg by intraperitoneal route).

Effect of Citrullus colocynthis in cell migration prevention: Cell count of sampled blood with EDTA is carried out using an automatic analyser (Rockwell). The influx of leukocytes into peritoneal cavities of mice with or without pretreatment was evaluated after washing the cavity with saline (NaCl 0.9%) after 30 min, 3 and 24 hours of envenomation.

Measurement of Myeloperoxidase Activity (MPO): Myeloperoxidase activity is evaluated as a marker of neutrophil infiltration. The myeloperoxidase activity was assayed by measuring the change in optical density at 460 nm using o-dianisidine and H_2O_2 (30V) diluted in phosphate buffer (50 mM; pH 7) [21].

Measurement of Eosinophil Peroxidase Activity: Collected mice sera are used in duplicate (50 µl/well of plate) in presence of chromogene substrate (20 mg of OPD solubilized in 10 ml of Tris HCl buffer - 0,05 M Triton X100 and 10 µl of H_2O_2), (100 µl/well). Reaction is incubated at 4°C during 1 hour in darkness. Absorbance was read at 490 nm [22].

Determination of C3 complement fraction: The antigen assay of C3 complement fraction was performed by nephelometry using commercial kits (The Binding Site, Birmingham, U.K).

Determination of C-reactive protein (CRP): CRP was measured by nephelometric method using a Beckman coulter analyzer.

Determination of blood glucose: Blood glucose rate was measured in all experiments using test strips at indicated times 30 min, 1, 2 and 3 hours after envenomation.

Determination of water content in the lungs of mice with or without pretreatment: Sampling of lungs is carried out 24 hours after envenomation. Lung is incubated at 37°C during 24 hours. The magnitude of pulmonary edema-forming was assessed by the ratio of wet to dry weight of different groups of animals. Dry and wet weights correspond respectively to the weight of lungs after incubation at 37°C during 24 hours and those just after sampling [23].

Evaluation of malondialdehyde activity MDA in mice with or without pretreatment: This assay is based on the formation of a complex between malondialdehyde and two molecules of thiobarbituric acid (TBA 0.6%) in a hot water bath (95C ° for 45 min). This complex can give a pink color that will be extracted by the organic solvent butanol after centrifugation (2000 g during 20 min). MDA concentration is determined by measuring the absorbance at 530 nm.

Determination of ASAT, ALAT and CPK in the serum: Determination of serum levels of the enzymatic activities of transaminases (ASAT and ALAT) and creatine phosphokinase (CPK) was carried out according to the manufacturer's instructions (RANDOX, SPINREACT).

Drugs: The following reagents were used: C3 fraction (The binding site, Birmingham, UK), ASAT and ALAT (Spinreact, Coloma, Girona, Spain), CPK (Randox, Crumlin, Antrim, UK).

Statistical analysis: Statistical analysis was performed using one way analysis of variance (ANOVA) and Tukey honest significant difference (HSD). Values of $P < 0.05$ were considered significant.

RESULTS:

Evaluation of the local action:

Evaluation of plantar edema induced by Aah venom on mice pretreated with Citrullus Colocynthis:

The results show an increase of paw edema volume in envenomed. However, significant decrease in a dose and time dependent was observed in pretreatment with *Citrullus Colocynthis*. Pretreatment with a dose of 50 mg/kg showed no significant difference with controls **(Figure 1).**

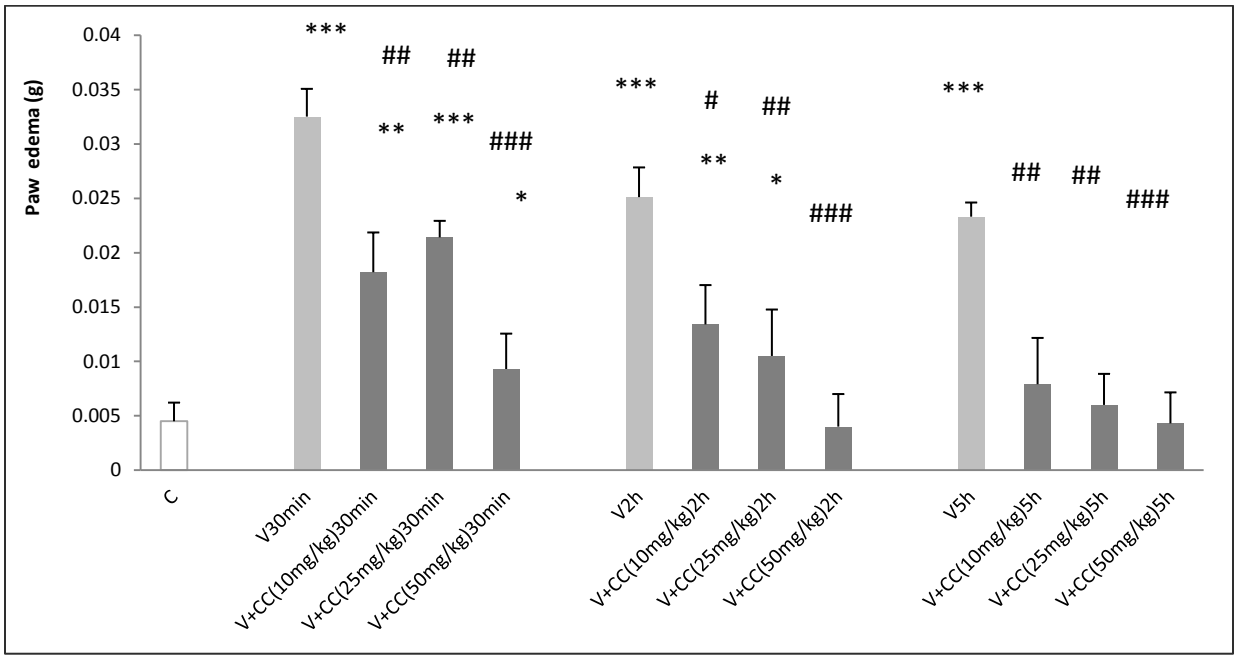

FIG. 1: Effect of *Citrullus colocynthis* (10 mg/kg, 25 mg/kg, 50 mg/kg) on the induced plantar Edema by Aah venom after 30 minutes, 2 and 5 hours. (C: control; V: venom; CC: *Citrullus colocynthis)*. *P < 0.05; **P < 0.01 and ***P < 0.001 compared to C (control); #P < 0.05; ##P < 0.01 and ###P < 0.001 compared to V (venom).

Evaluation of systemic action:

Effect of Citrullus colocynthis on cell migration: The immune cell count in the blood and after washing the peritoneal cavity of pretreated mice with *Citrullus colocynthis*, 3 and 24 hours after envenomation is almost similar to that of controls, suggesting a preventive effect of *Citrullus colocynthis* in cell migration **(Figures 2 and 3).**

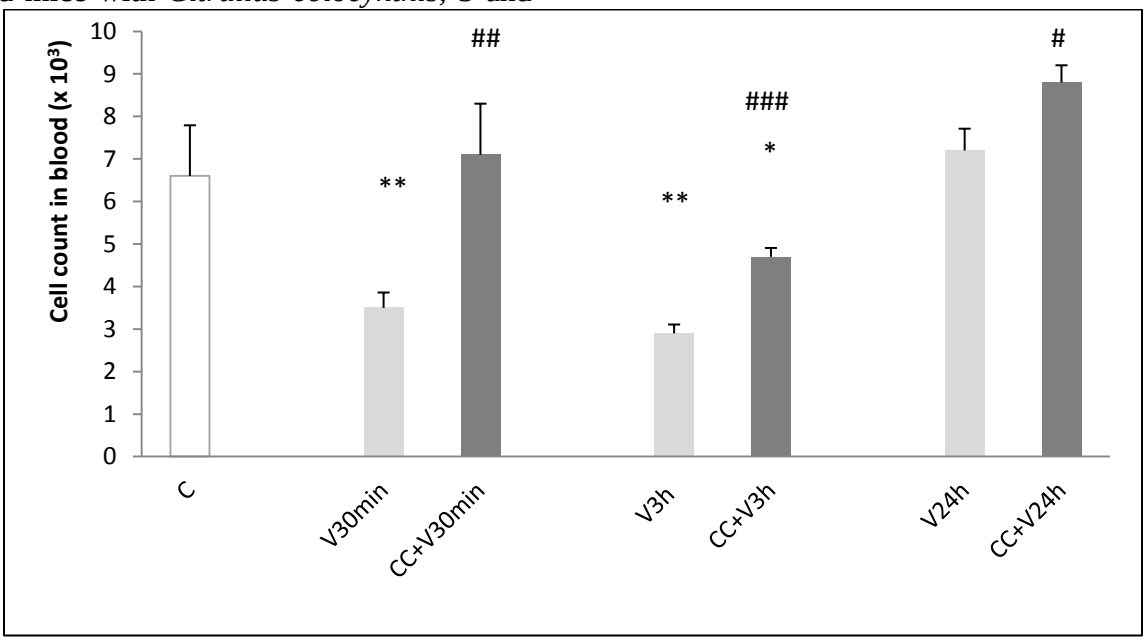

FIG. 2. Effect of *Citrullus colocynthis* in the prevention of cell migration induced by the Aah venom (10 µg/20 g body weight) in blood after 30 min, 3 and 24 hours of envenomation (C: control, V: venom, CC: *Citrullus colocynthis*). *P < 0.05; **P < 0.01 compared to C (control); #P < 0.05; ##P < 0.01 compared to V (venom).

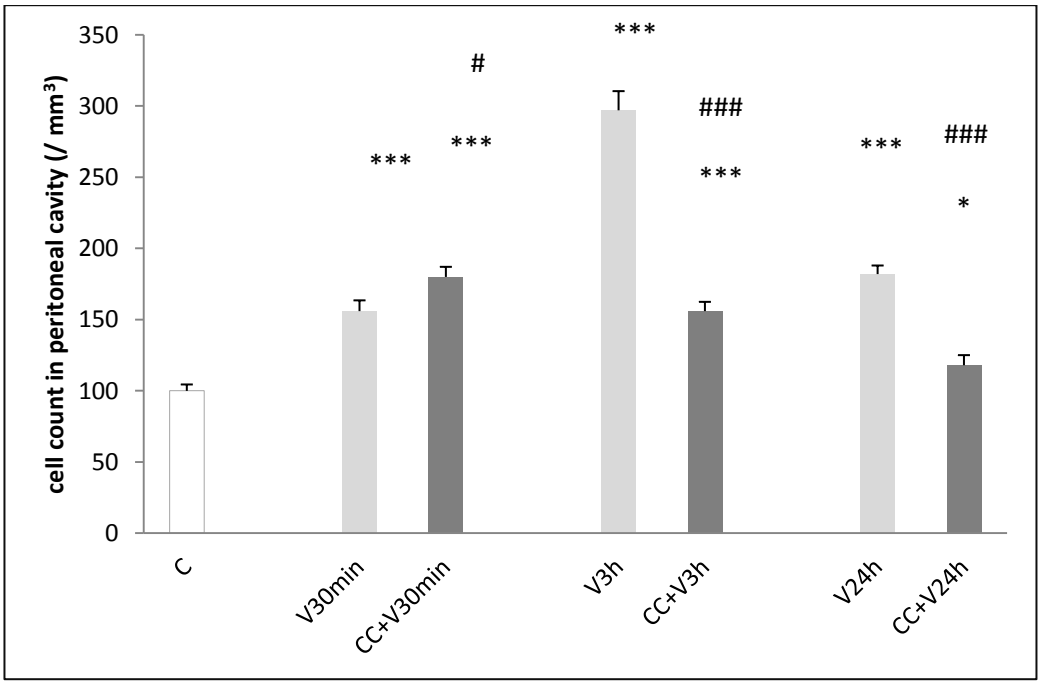

FIG. 3. Effect of *Citrullus colocynthis* in the prevention of cell migration induced by the Aah venom (10 µg/20 g body weight) into the peritoneal cavity after 30 min, 3 and 24 hours of envenomation (C: control, V: venom, CC: *Citrullus colocynthis*). *P < 0.05; **P < 0.01 compared to C (control); #P < 0.05; ##P < 0.01 compared to V (venom).

Evaluation of myeloperoxidase activity (MPO): High increase of MPO level was observed mainly 3 hours after envenomation, this activity is slightly elevated in pretreated mice with *Citrullus colocynthis* after 30 min of envenomation. It becomes similar to that of control mice after 3 and 24 hours **(Figure 4).**

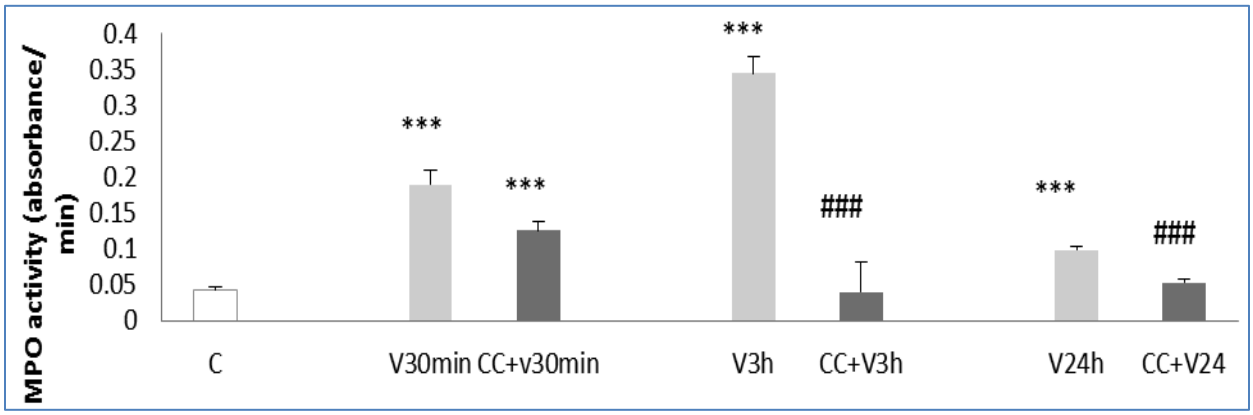

FIG. 4: Determination of MPO activity in the serum after 30 min, 3and 24 hours of envenomation by the Aah venom (10 µg/20 g body weight) with or without pretreatment with *Citrullus colocynthis* (C: control, V: venom, CC: *Citrullus colocynthis*). *P < 0.05; **P < 0.01 and ***P < 0.001 compared to C (control); #P < 0.05; ##P < 0.01 and ###P < 0.001 compared to V (venom).

Evaluation of eosinophil peroxidase activity (EPO):

They showed a High EPO level was observed in sera of envenomed mice mainly after 3 hours this level was lower observed in mice pretreated with *Citrullus colocynthis* **(Figure 5).**

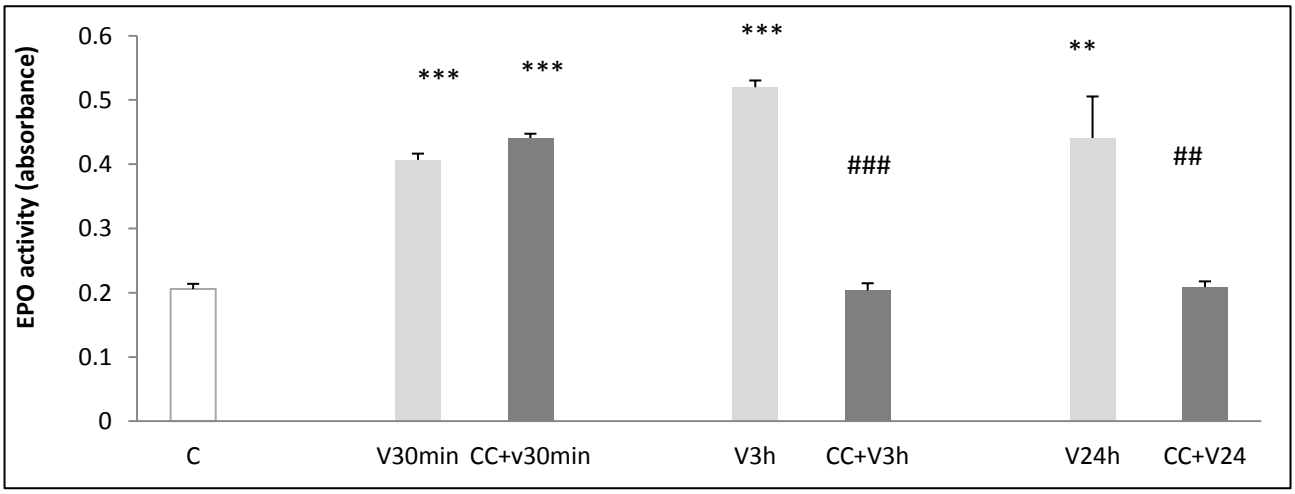

FIG. 5: Determination of EPO activity in the serum after 30 min, 3 and 24 hours of envenomation by the Aah venom (10 µg/20 g body weight) with or without pretreatment with *Citrullus colocynthis* (C: control, V: venom, CC: *Citrullus colocynthis*). *P < 0.05; **P < 0.01 and ***P < 0.001 compared to C (control) ; #P < 0.05; ##P < 0.01 and ###P < 0.001 compared to V (venom).

Determination of C3 complement fraction: Without pretreatment, a sublethal dose of Aah venom injected by intraperitoneal route induced 30 minutes and 24 hours an increase in serum C3 complement fraction **(Figure 6).** However, pretreatment of animals with *Citrullus colocynthis* seems to prevent the increase of serum C3 fraction observed in untreated animals **(Figure6).**

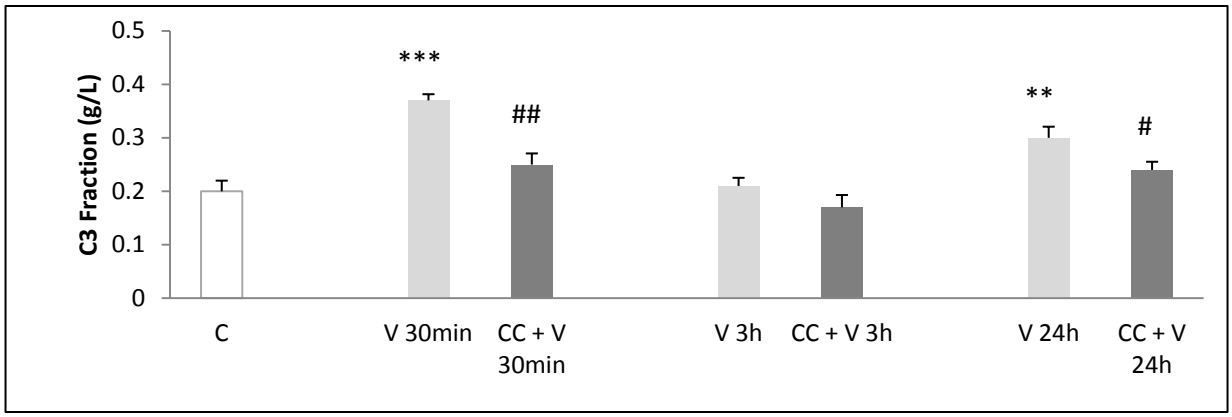

FIG. 6: Determination of serum C3 after 30 min, 3and 24 hours of envenomation by the Aah venom (10 µg/20 g body weight) with or without pretreatment with *Citrullus colocynthis* (C: control, V: venom, CC: *Citrullus colocynthis*). *P < 0.05; **P < 0.01 and ***P < 0.001 compared to C (control); #P < 0.05; ##P < 0.01 and ###P < 0.001 compared to V (venom).

Determination of C-reactive protein (CRP): Aah venom injected to animals significantly increased the serum level of CRP **(Figure 7).** Pretreatment with *Citrullus colocynthis* decreased the level of CRP even after 3 hours as in control mice **(Figure 7).**

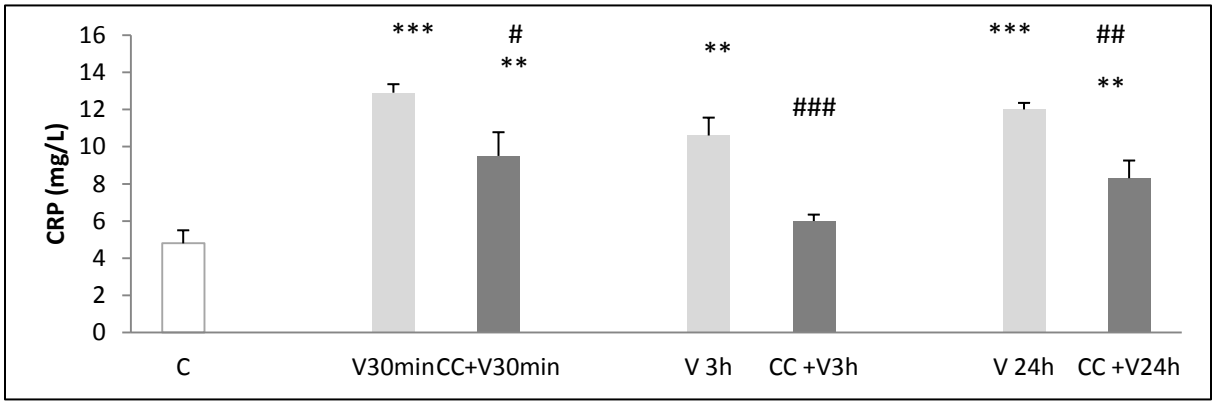

FIG. 7. Determination of serum CRP after 30 min, 3and 24 hours of envenomation by the Aah venom (10 µg/20 g body weight) with or without pretreatment with *Citrullus colocynthis* (C: control, V: venom, CC: *Citrullus colocynthis*). *P < 0.05; **P < 0.01 and ***P < 0.001 compared to C (control); #P < 0.05; ##P < 0.01 and ###P < 0.001 compared to V (venom).

Determination of blood glucose: Injected animals with Aah venom showed hyperglycemia (**Figure 8**). Blood glucose level decreased in pretreated animals with *Citrullus colocynthis*, (Figure 8).

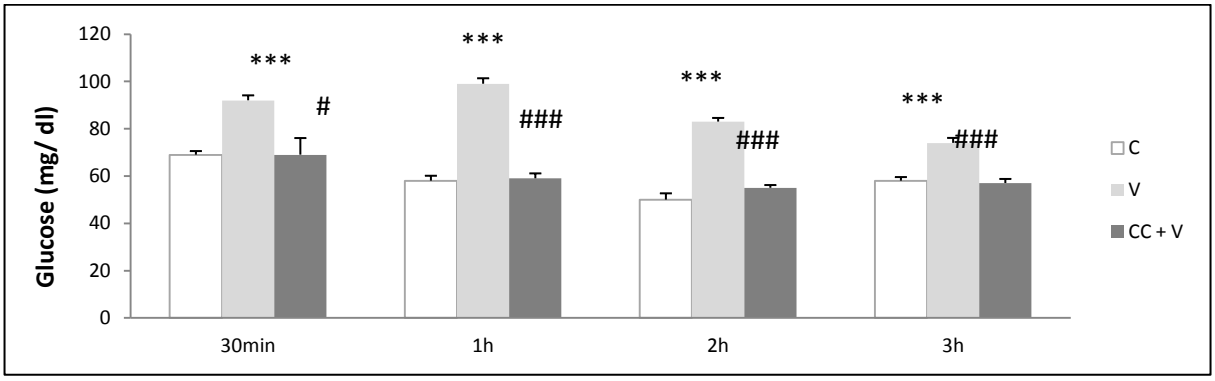

FIG. 8. Effect of *Citrullus colocynthis* in the prevention of hyperglycemia induced by the Aah venom (10 µg/20 g body weight). after 30 min, 2 and 3 hours (C: control, V: venom, CC: *Citrullus colocynthis*). *P < 0.05; **P < 0.01 and ***P < 0.001 compared to C (control) ; #P < 0.05; ##P < 0.01 and ###P < 0.001 compared to V (venom).

Evaluation of the induced pulmonary edema by Aah venom on pretreated mice with Citrullus colocynthis: The extent of edema formed in lungs was assessed 30 minutes, 3 and 24 hours after envenomation of pretreated mice with *Citrullus colocynthis* (50 mg/kg) using the ratio between the wet and dry weight of organs. An increase of the ratio between the wet and dry weight of the lungs of untreated mice increased significantly after envenomation. However, mice pretreated with *Citrullus colocynthis* show a slight increase **(Figure 9).**

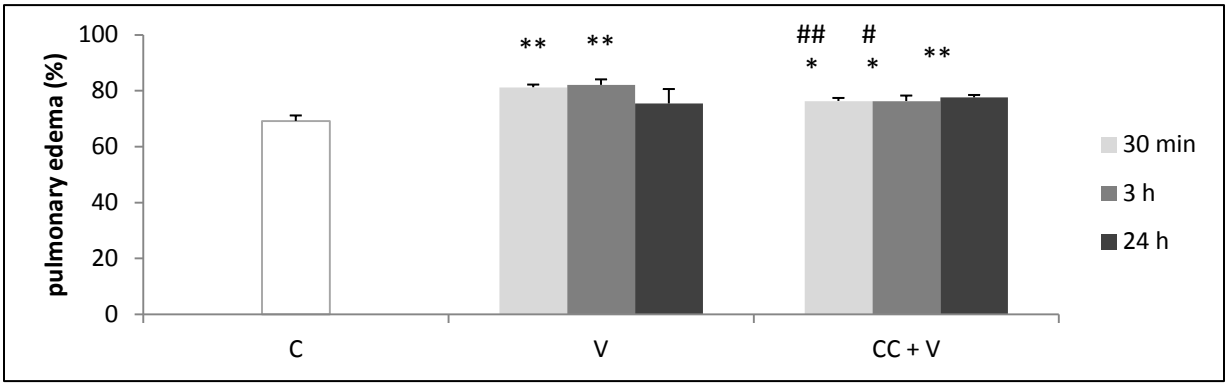

FIG. 9. Evaluation of induced pulmonary edema by Aah venom (10 µg/20 g body weight) after 24 hours with or without pretreatment with *Citrullus colocynthis* (C: control, V: venom, CC: *Citrullus colocynthis*). *P < 0.05; **P < 0.01 compared to C (control); #P < 0.05; ##P < 0.01 compared to V (venom).

Evaluation of malondialdehyde activity (MDA) in mice with or without pretreatment: The results showed a significant increase in serum levels of MDA after 30 min in envenomed mice which returns its normal after 24 hours **(Figure 10).** When animals were pretreated with *Citrullus colocynthis*, MDA levels remains similar to that of control mice (Figure 10).

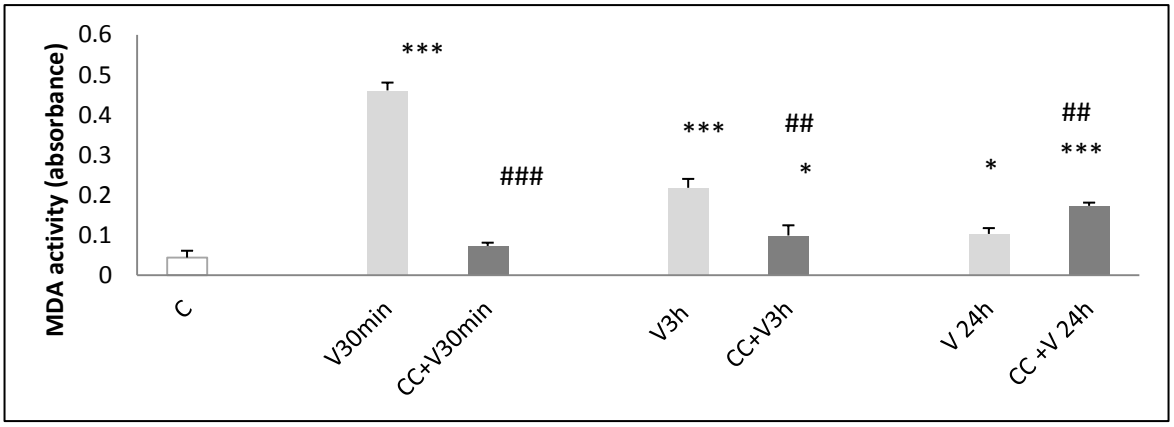

FIG. 10. Evaluation of serum MDA activity after 30 min, 3 and 24 hours of envenomation by the Aah venom (10 µg/20 g body weight) pretreated or no with *Citrullus colocynthis* (C: control, V: venom, CC: *Citrullus colocynthis*). *P < 0.05; **P < 0.01 and ***P < 0.001 compared to C (control); #P < 0.05; ##P < 0.01 and ###P < 0.001 compared to V (venom).

Determination of ASAT, ALAT and CPK in the serum: Obtained results showed an increase in serum enzymatic activities of ASAT, ALAT and CPK of envenomed animals **(Figure 11).** However when mice were pretreated with *Citrullus colocynthis* and then envenomed, these activities decreased at 24 hours.

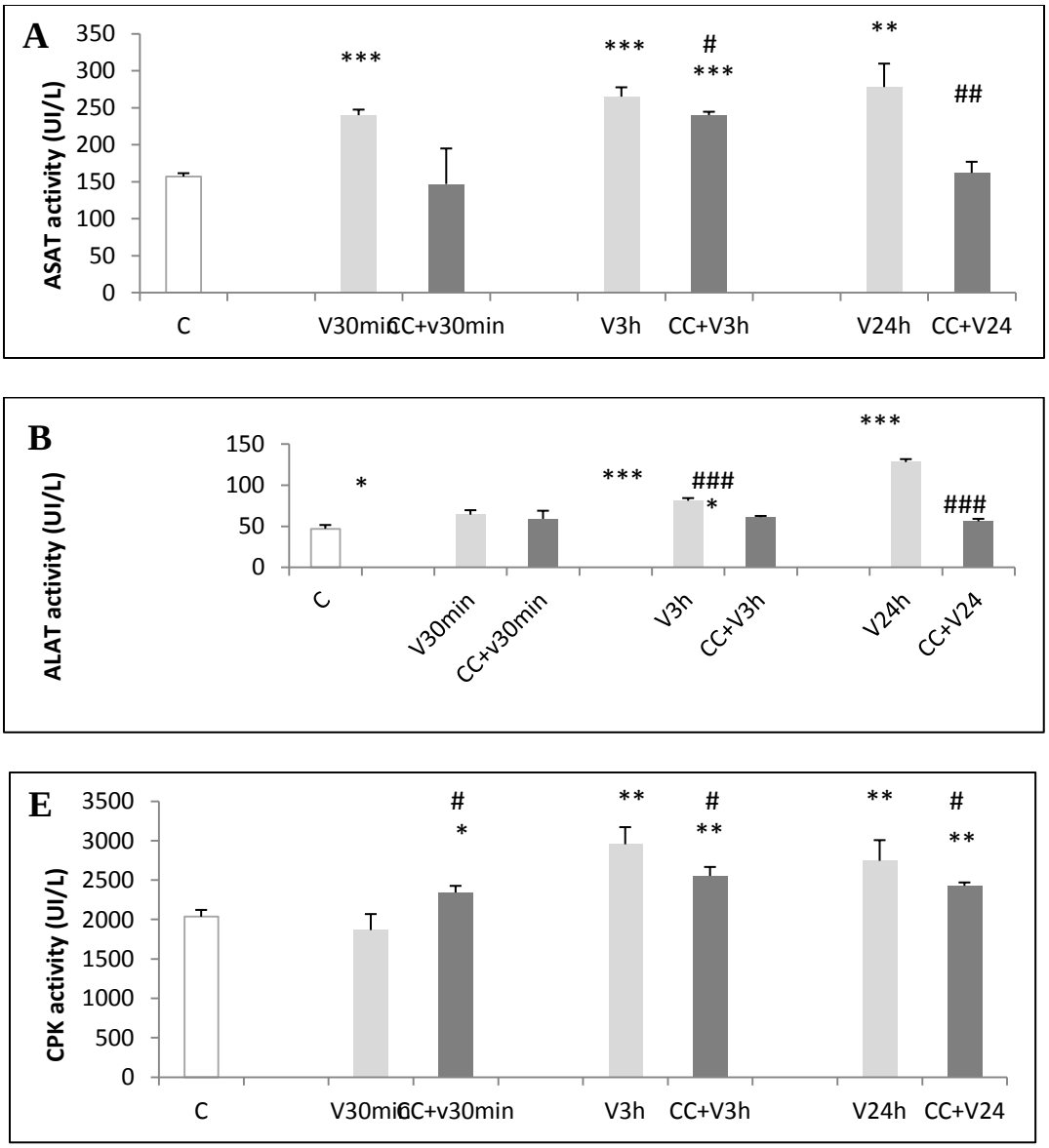

FIG. 11: Determination of serum (A: ASAT, B: ALAT and E: CPK) activity after 30min, 3 and 24 hours of envenomation by the Aah venom (10 µg/20 g body weight) with or without pretreatment with *Citrullus colocynthis*. (C: control, V: venom, CC: *Citrullus colocynthis*). *P < 0.05; **P < 0.01 and ***P < 0.001 compared to C (control); #P < 0.05; ##P < 0.01 and ###P < 0.001 compared to V (venom).

DISCUSSION: Our findings suggest that scorpion envenomation induces systemic and local inflammation. We observed increased plantar edema in untreated mice with *Citrullus colocynthis.* The increased vascular permeability and edema formation are early events in the inflammatory response followed by leukocyte infiltration. The pretreatment of mice by *Citrullus colocynthis* prevents the induced plantar edema. Plantar edema decrease is due to anti-inflammatory and anti-histaminic properties of *Citrullus colocynthis*, and to its action on serotonin and kinin-like substances by *Citrullus colocynthis* [24, 10, 2, 3].

These effects are probably due in part to the decreased production of pro-inflammatory cytokines (IL-1b, IL-6) and expression of COX-2 [24]. IL-1b inhibition reduces the expression of chemokines and adhesion molecules by endothelial cells. The Similar results showed that intraperitoneal injection of aqueous extracts of reproductive organs (fruits and seeds) of *Citrullus colocynthis* significantly reduced plantar edema induced by carrageenan (1%). This reduction due to anti-inflammatory properties of this plant is variable and depends on the dose of the administered extract [10].

C-reactive protein is an acute phase protein that appears in the blood during inflammatory processes. It rises rapidly to become an early marker of the inflammatory response. The scorpion envenomation in our study significantly increased serum CRP. Similar results have shown that the C-reactive protein (CRP) increases after scorpion envenomation [25]. However, the pretreatment with *Citrullus colocynthis* decrease serum CRP. This decrease can be attributed to the reduction of the production of pro-inflammatory cytokines (IL-6 and IL-1) [24] which play an important role in stimulating the production of CRP in hepatocytes. Similar results show that the administration of *Cucurbita pepo* in diabetic rats significantly reduced CRP levels in the blood compared to diabetic control [26].

We observed increased in blood glucose level after Aah envenomation. Cytokines release after scorpion envenomation play a role in the development of hyperglycemia, particularly IL-1β, TNF-α, IFN-γ which are putative mediators of progressive loss of β cells in the pancreas and the development of insulin resistance [27]. However, the pretreatment with *Citrullus colocynthis* decrease blood glucose level. Several mechanisms have been proposed on the hypoglycemic property of *Citrullus colocynthis*. This hypoglycemic property is probably due to the increased production of insulin, decreased gluconeogenesis and increased glycolytic activity [28, 29]. In addition, the extraction products of *Citrullus colocynthis* showed similar structure to animal insulin on electrophoresis and infrared spectrophotometer which may lead to a decrease in blood glucose level [30]. Similar results indicate that administration of different concentrations of *Citrullus colocynthis* powder significantly reduced the rate of blood glucose in diabetic models (rats) induced by streptozotocin [31, 32].

Scorpion envenomation induces an increase in serum ASAT and ALAT which may be due to a direct action of the venom on the liver and heart [27]. It was reported that *Citrullus colocynthis* is able to decrease serum alanine aminotransferase (ALAT) [33]. In our study, *Citrullus colocynthis* decrease the activity of ASAT, ALAT and CPK in the serum.

Another marker of oxidative stress Malondialdehyde (MDA) produced by cellular peroxidation under the action of free fatty acids, significant increased by scorpion venom which also inhibit the activity of glutathione, superoxide dismutase (SOD) and catalase (CAT), with a significant increase in the level of MDA [34]. Pretreatment with *Citrullus colocynthis* show a protective effect against oxidative stress. This effect is related to the increase of glutathione, superoxide dismutase after administration of *Citrullus colocynthis*, it play a protective role against oxidative stress [35]. According to Kumar et al (2008) [36] the *Citrullus colocynthis* contains flavonoids phenolics and products with anti-oxidant activity. Similar results show that cucurbitaceae reduced MDA levels in serum and increase the superoxyde dismutase and glutathione peroxidase activity due to its antioxidant capacity [37]. Other results confirm the antioxidant activity of *Citrullus colocynthis* [4, 5].

CONCLUSION: In conclusion, *Citrullus colocynthis* can be used as a symptomatic treatment in stung patients especially in rural environments to decrease the severity of pathophysiological effects induced after envenomation (inflammation and oxidative stress) all the more than *Citrullus colocynthis* is available and easy to prepare.

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