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Rosemary leaves extract: Anti-snake action against Egyptian *Cerastes cerastes* venom

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ABSTRACT

The morbidity caused by viper bites is very dangerous and the anti-venom therapy couldn't treat the local injures such as hemorrhage, edema, necrosis and inflammation of bitten tissues. Searching for safe and effective anti-venom compounds from natural sources is very important. This study was designed to explore the neutralizing ability of *Rosmarinus officinalis* L. leaves aqueous extract (RMAE) against Egyptian *Cerastes cerastes* (Cc) viper venom toxicity. The RMAE contained a considerable amount of phenolic and flavonoid contents with 3,300 and 800 mg/100 g dry weight, respectively. The RMAE showed a considerable variation of phenolic acids by using HPLC technique. Rosmarinic acid is the major component of the RMAE which recorded 400 mg/100 g dry weight and 64% of all the identified compounds. In vitro, the RMAE neutralized the enzymatic activities of proteases, L-amino acid oxidases, and phospholipases A_2 of the Cc venom dose-dependently. In addition, the RMAE effectively neutralized the gelatinolytic, fibrinogenolytic, hemolytic and procoagulant activities of Cc venom. In vivo, the RMAE markedly reduced lethality, hemorrhage, edema, muscle and liver toxicities induced by Cc venom. In conclusion, the venom neutralizing property of the RMAE gives a new prospect for efficient treatment of the lethal viper bites.

1. Introduction

Snake bite is still a common medical human health problem in many tropical and subtropical areas of the world, especially in northern Africa, southern Asia, Latin America and Middle East resulting in high mortality and morbidity.[1] More than 90% of snake venom composition is toxic proteins, which includes mainly the enzymes of phospholipases A_2, hemorrhagic metalloproteases, L-amino acid oxidases and hyaluronidases. These enzymes implicated in a series of dangerous events happened in the victim's body following the envenomation.[2] The Egyptian desert horned viper (*Cerastes cerastes*) is the most distinctive and plenty viper of North Africa and the Middle East deserts. Envenomings by *Cerastes cerastes* viper caused local tissue injuries, such as swelling, blistering, hemorrhage and necrosis of skeletal muscles, which occasionally leading to amputation of the injured limb.[3]

Anti-venoms are the only specific medication of snake bites; however, they have many disadvantages such as high cost, hypersensitivity, non-availability and their limitation in the neutralization of local tissue damage. Degradation of local tissue damage is a nonstop process which is prolonged even after the administration of anti-venoms.[4] So, the searching for supplementary/alternative snake bite remedies is fundamental.[5] Plant extracts have an amazing mixture of diverse active compounds. These compounds possess diverse pharmacological activities which they can bind with the toxins/enzymes leads to the neutralization/inhibition of their activities.[6]

Rosemarinus officinalis L. (rosemary) is an evergreen perennial aromatic shrub belonging to the family *Lamiaceae*. Previously, rosemary was grown in the north and south coasts of the Mediterranean Sea but now it is cultivated all over the world as an aromatic plant. Rosemary leaves are used for food flavoring and in folk medicine such as antispasmodic, dysmenorrhoea, relieving respiratory disorders and heart diseases, analgesic, antirheumatic, carminative, cholagogue, diuretic, antiepileptic, stimulate the growth of hair and treatment of eczema of the scalp, boils and

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wounds.[7–9] It also had anti-proliferative effect against cancer cells, high antioxidant, antimicrobial, anti-inflammatory, anti-diabetogenic and hepatoprotective activities.[10–13] Previously, we demonstrated that the rosemary leaves aqueous extract had potent hyaluronidase inhibitory effect among several medicinal plants tested in this study.[14] From this standpoint, the present study aims to evaluate the anti-venom properties of rosemary leaf aqueous extract against Egyptian *Cerastes cerastes* viper venom toxicity using both in vitro and in vivo assays.

2. Materials and methods

2.1. Plant collection

Rosmarinus officinalis L. (rosemary) plant was collected from Horticulture Institute Research, Agriculture Research Center (ARC), Cairo, Egypt. It was authenticated by Dr. M. Salah (Professor, Botany department, ARC, Cairo, Egypt). A voucher specimen was deposited in the herbarium of the same department under the number ARC/42014.

2.2. Plant extraction

Rosemary leaves were thoroughly washed with water, shade-dried and grounded into coarse powder. Five grams of rosemary leaves powder were soaked in 20 ml distilled water (w/v) overnight at room temperature with constant stirring. The obtained extract was filtered using Whatman filter paper No. 1. Then, the extract was centrifuged, lyophilized and dissolved in least volume of 0.1 % DMSO and designated as rosemary aqueous extract (RMAE).

2.3. Animals

Male Swiss-albino mice weighing 20 ± 1 g were used for this study. All animals were maintained at the animal house, National Research Centre (NRC), under standard conditions and diet. Experiments were carried out with prior permission from the Institutional Animal Ethical Committee, NRC, Egypt.

2.4. Snake venom and chemicals

Cerastes cerastes (Cc) venom was milked from adult vipers which collected from El-Faiyum, Egypt. Directly after extraction, the venom was pooled, divided in aliquots, lyophilized, and stored at −20 °C. Azocasein, tricholoroacetic acid, Tris base, acrylamide, bis-acrylamide, fibrinogen, gelatin, coomassie brilliant blue R-250, L-Leucine and o-phenylenediamine (OPD) were obtained from sigma. ALT and AST diagnostic kits were purchased from Biodiagnostic Co., Egypt. All other chemicals and reagents were of analytical grade. The buffers were prepared according to Gomorie[15] and the final pH was checked by pH meter (Hanna, pH 211 Microprocessor pH meter).

2.5. Total phenolic content estimation

The total phenolic content was measured according to Velioglu et al.[16] Fifty μL of the rosemary aqueous extract (RMAE) was mixed with 100 μL Folin–Ciocalteu reagent, 850 μL of distalled water and allowed to stand for 5 min at ambient temperature. A 500 μL of 20% sodium carbonate was added and the mixture was incubated for 30 min at room temperature. Absorbance was measured at 750 nm. Gallic acid was used as standard for the calibration curve and total phenolic content was expressed as mg gallic acid equivalent (GAE)/100 g dry weight.

2.6. Total flavonoid content estimation

The total flavonoid content was determined using modified colorimetric method described previously by Zhishen et al.[17] and used catechin as a standard. RMAE (250 μL) was mixed with 1.25 μL distilled water and 75 μL of 5 % $NaNO_2$ solution. After 6 min, 150 μL of 10% $AlCl_3$ solution was added to the mixture. 0.5 ml of 1 M NaOH and 275 μL of distilled water were added to the mixture 5 min later. The absorbance of the solutions was measured at 510 nm. The results were expressed as mg catechin equivalent (CE)/100 g dry weight.

2.7. HPLC analysis of phenolic compounds

The high performance liquid chromatography (HPLC) analysis was carried out for RMAE according to Kim et al.,[18] using an Agilent Technologies 1100 series liquid chromatography equipped with an auto sampler and a diode-array detector. The separation and determination were performed on XDB- C18 column (150 × 4.6 μm). The mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The total run time was 70 min at flow rate one ml/min with gradient programmed as follows: 100 to 85% (in 30 min), 85 to 50% (in 20 min), 50 to 0% (in 5 min) and 0 to 100% (in 5 min) of solvent B. There was 10 min of post-run for reconditioning. The obtained peaks were monitored simultaneously at 280, 320 and 360 nm. All samples were filtered through a 0.45 μm Acrodisc syringe filter (Gelman Laboratory, MI) before injection. Peaks were identified by congruent retention times and UV spectra and compared with the standards (Fig. 1A and B).

2.8. Protease inhibition

2.8.1. Azocasein assay

The caseinolytic activity was determined according to Lemos et al.[19] with slight modifications. One ml assay reaction mixture containing 20 mM Tris-HCl buffer, pH 8.5, 0.2% azocasein and 5 μg of crude venom was incubated 1 h at 37 °C. The reaction was stopped by the addition of 0.1 ml of 10% (TCA), followed by centrifugation at 5,000×*g* for 5 min and the absorbance was recorded at 366 nm. One unit of proteolytic activity was defined as the increase in the absorbance at 366 nm of 0.1 after 1 h in the test reaction compared with the control reaction. For inhibition studies, 5 μg of the crude venom was pre-incubated with various ratios of RMAE (1:0, 1:2, 1:4, 1:6, 1:8 and 1:10) for 30 min at 37 °C and the residual activity was measured according standard assay condition.

2.8.2. Gelatin zymography

Gelatin Zymogram was performed in 12% native polyacrylamide gel containing 2 mg/mL gelatin according to Bee et al.[20] The conditions of electrophoresis were carried out according to Laemmli.[21] Then, the gel was incubated overnight at 37 °C in 20 mM Tris -HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4. The gel was stained by 0.1% Coomassie brilliant blue in a solution of methanol: acetic acid: water in 45:10:45 (v: v: v) and the de-stained by the same solution. For inhibition experiments, 30 μg of the crude venom was pre-incubated with different ratios of RMAE (1:2.5, 1:5, 1:10 and 1:20) for 30 min at 37 °C then performed according to the method previously described.

2.9. L-amino acid oxidase inhibition

The microplate assay for L-amino acid oxidase (LAAO) activity was conducted as described by Kishimoto and Takahashi[22] with slight modifications. The reaction mixture contained 50 mM of

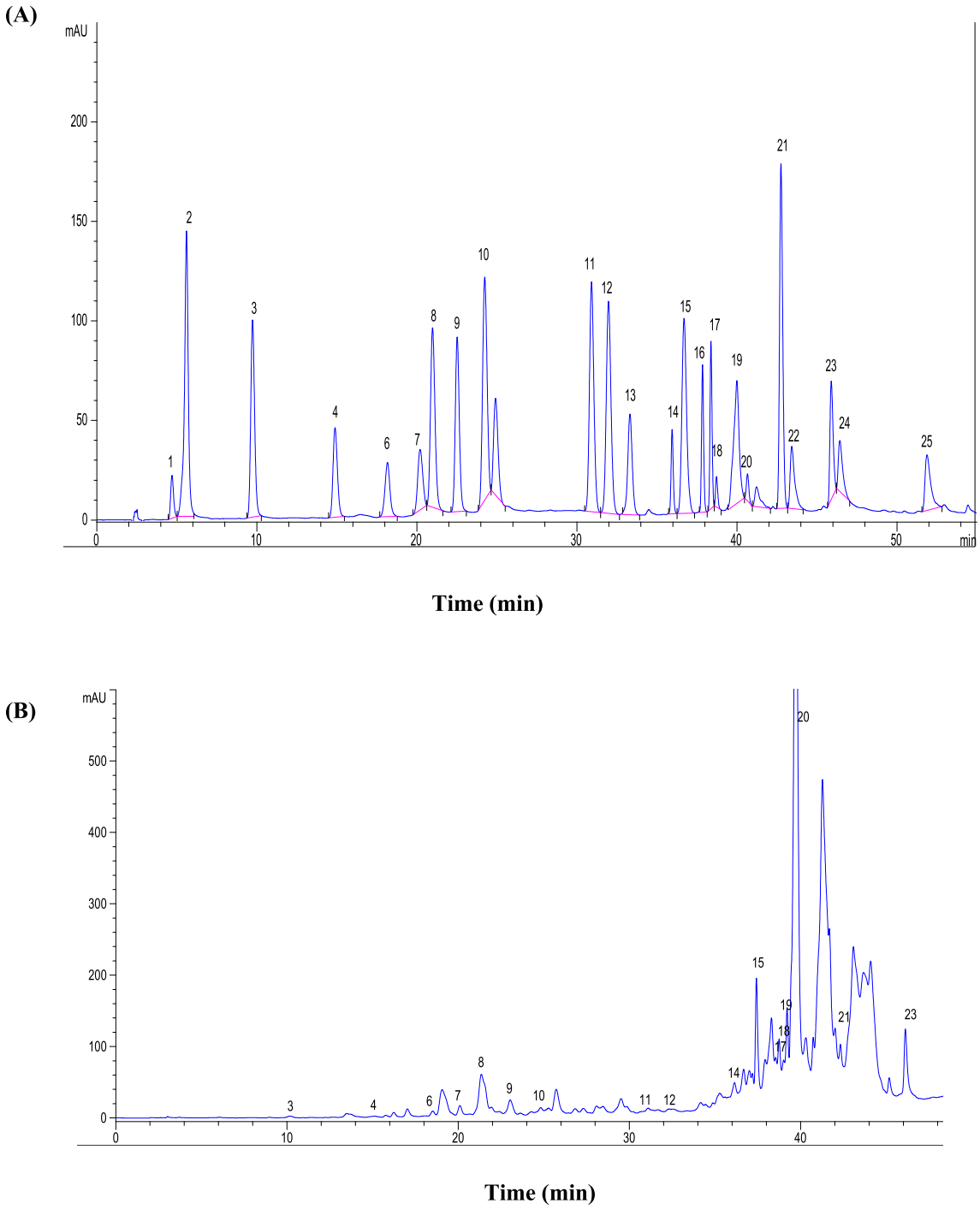

Fig. 1. (A) HPLC profile of Phenolic compounds as standards at 280 nm. (1) pyrogallol, (2) gallic, (3) protochatchuic, (4) p-hydroxybenzoic, (5) gentisic, (6) catachine, (7) chlorgenic, (8) caffic, (9) syrngic, (10) vanillic, (11) scoplatine, (12) ferulic, (13) sinapic, (14) rutin, (15) p-coumaric, (16) naringeen, (17) hisperdin, (18) apeginin-7-glucoside, (19) myrcetin, (20) rosmarinic, (21) cinnamic, (22) qurcetin, (23) apegnin, (24) kaempferol, and (25) chyrsin. (B) HPLC profile of the RMAE at 280 nm. The major peak is at R_{time} of 40 min corresponding to rosmarinic acid (RA).

Tris–HCl buffer, pH 8.0, 5 mM L-leucine as substrate, horseradish peroxidase (5 IU/mL) and 2 mM orthophenylenediamine (as substrate for peroxidase). Venom Sample (2 μg) was incubated for 1 h at 37 °C and the reaction was stopped by adding 50 μL of 2N H_2SO_4. The absorbance was recorded at 490 nm by ELISA reader. One unit of LAAO activity was the amount of enzyme which produces 1 μmol of H_2O_2. The inhibition experiments were performed by pre-incubated 2 μg of Cc venom with different ratios of RMAE (1:0, 1:2, 1:4, 1:6, 1:8, and 1:10) for 30 min at 37 °C. The residual activity was measured according standard assay condition. Crude venom was considered as 100% activity.

2.10. Phospholipase A2 (PLA_2) inhibition

PLA_2 activity was determined using egg yolk as PLA_2 substrate (lecithin) according to Marinetti.[23] Briefly, the assay was carried out

by using egg yolk suspension diluted in saline (1:5 w/v). Crude venom sample (200 μg) alone or pre-incubated with different ratios of RMAE (1:0, 1:1, 1:2, 1:3, and 1:4) for 30 min at 37 °C was added to one ml of egg yolk working suspension to a final assay mixture volume of 5 ml with saline and the absorbance was recorded each 5 min for 15 min at 900 nm.

PLA_2 activity was also carried out according to Gutierrez et al.[24] Briefly, 200 μg of crude Cc venom was loaded into 3 mm diameter wells of 1% agarose plates containing 4% washed human erythrocytes, 4% egg yolk suspension and 10 mM $CaCl_2$. The fortified agarose-egg yolk plate was incubated for 20 h at 37 °C. The PLA_2 inhibition was calculated by measuring the zone of clearance around the haloes in the presence and absence of venom/RMAE ratios (1:0, 1:1, 1:2, 1:3, 1:4, and 1:5). PLA_2 activity of venom in the absence of RMAE served as positive control.

2.11. Hemolytic inhibition

The hemolytic activity was evaluated according to the method of Abdulla et al.[25] Briefly, a pre-warmed assay mixture containing 100 μl of egg yolk suspension (diluted 1:5 in 0.9% isotonic saline), 1 ml of washed human erythrocytes [(2.5% (v/v)], 3.8 ml of hemolytic buffer (1 mM Tris-HCl buffer, pH 7.5 containing 100 mM NaCl, 100 mM KCl and 10 mM $CaCl_2$) and crude venom (5 μg) alone or pre-incubated with different ratios of RMAE (1:0, 1:5, 1:10, 1:20, and 1:30) for 30 min at 37 °C. The reaction was centrifuged at 2500 rpm for 10 min and the amount of hemoglobin released in the supernatant was estimated at 540 nm.

2.12. Coagulant inhibition

The plasma coagulation activity was assayed according to Angulo et al.[26] Two hundred μl of platelet poor human citrated plasma was incubated with 1 μg of Cc venom in 0.05 M phosphate buffer saline, pH 7.5 for 10 min at 37 °C. Then, 20 μl of 0.25 M $CaCl_2$ was added and the clotting time was recorded. The inhibition experiments were performed by pre-incubated venom sample with different ratios of RMAE (1:0, 1:2.5, 1:5, 1:10, and 1:20) for 30 min at 37 °C and the clotting time in sec was recorded.

2.13. Fibrinogen degradation

Fibrinogenase activity was assayed according to Ouyang and Teng.[27] The plasma fibrinogen (2 mg/ml) in 5 mM Tris-HCl buffer, pH 7.5, containing 10 mM NaCl was mixed and incubated with 5 μg of the venom at 37 °C for 2 h. Then, 0.1 ml of the incubation solution was added to 0.1 ml of stopping solution (10M urea, 4% SDS, and 4% 2-mercaptoethanol). The samples were analyzed on 10% SDS-PAGE according to Laemmli.[21] The inhibition experiments were performed by pre-incubated venom sample with ratios of RMAE (1:0, 1:10, 1:20 and 1:40) for 30 min at 37 °C, and then performed according to the method previously described.

2.14. Neutralization of lethal potency of Cc venom

The median lethal dose (LD_{50}) of Cc venom was determined according to the method of Meier and Theakston.[28] Groups of 5 mice were injected intraperitonially (i.p) with different concentrations of venom (2–32 μg protein/mouse) in 0.5 ml of saline. Then, the survival time of each animal in each set was recorded for 24 h. LD_{50} was calculated by the comparison of doses injected, with the observed survival time within 24 h. In neutralization studies, eight groups (n = 5), the groups from 1 to 4 were injected i.p with doses of the 1/2 LD_{50}, 1 LD_{50}, 2 LD_{50} and 4 LD_{50} of Cc venom alone respectively. Whereas, groups from 5 to 8 were injected with 1/2 LD_{50}, 1 LD_{50}, 2 LD_{50} and 4 LD_{50} venom, respectively and after 10 min following venom injection 0.5 mg/ml of RMAE were injected i.p. Two control groups were received either saline or 0.5 mg of RMAE, respectively. The survival time was recorded.

2.15. Neutralization of hemorrhagic activity

Hemorrhagic activity was determined according to the method of Kondo et al.[29] using seven groups (n = 5) of mice. The samples were injected dorsally and intradermally in 100 μl of saline. Briefly, the first group received 1 μg (2 MHD) of Cc venom alone and groups 2, 3, 4, 5 received the pre-incubated venom sample (2 MHD) with different ratios of RMAE (1:5, 1:10, 1:20 and 1:40), respectively for 30 min at 37 °C. Groups 6 and 7 received either RMAE or saline as controls. Skins were removed after 2 h and the diameters of the hemorrhagic spots were measured on the inside surfaces.

2.16. Neutralization of edema activity

Edema inducing activity was carried out according to the method of Vishwanath et al.[30] Six groups of five mice were injected intramuscular into the right footpads with final volume of 100 μl saline. Group 1 was injected with 20 μg (5 MED) of Cc venom, groups 2, 3, 4, 5 were injected with pre-incubated different ratios of venom: RMAE (w/w) (1:5, 1:10, 1:20 and 1:40), respectively for 30 min at 37 °C. The sixth group was injected with 0.25 mg/ml of RMAE alone and the left footpads received equal volume of saline as controls. The footpad thickness was measured after 1 h and the MED defined as amount of venom required to cause edema ratio of 120%.

2.17. In vivo neutralization of myotoxicity of Cc venom

The myotoxicity was determined according to the method of Gutierrez et al.[31] Four groups of five mice were used in absence and presence of RMAE. Group 1 was injected intramuscularly with $2LD_{50}$ of Cc venom alone. Group 2 injected with 0.5 mg/ml of RMAE after 10 min of the venom injection. Groups 3 and 4 were injected with saline and RMAE as control, respectively. The mice were anesthetized by diethyl ether inhalation after 1 h and the abdominal cavities were opened to draw the blood from vena cava. A piece of skeletal muscle tissue at the venom injected spot was taken and immediately processed for histopathological studies. The collected serum was assayed for creatine phosphokinase (CPK, EC 2.7.3.2) activity using diagnostic kit. Activities were expressed as IU/L.

2.18. Histo-pathological studies in mice

Four groups of mice (n = 5) were injected i.p. for pathological study. Group 1 received $2LD_{50}$ of Cc venom alone and group 2 was injected after 10 min of venom injection with 0.5 mg/ml of RMAE. Groups 3 and 4 received RMAE and saline alone, respectively, as control. The mice were sacrificed by cervical dislocation and the liver was taken, fixed in 10% formalin solution and dehydrated in a grade alcohol series 24 h and then embedded in paraffin wax. Sections of 4–5 μm thickness were stained with hematoxylin–eosin for pathological studies as described by Kiernan.[32] The photographs were taking using digital camera attached to the microscope. In addition, the collected serum was assayed for ALT (EC 2.6.1.2) and AST (EC 2.6.1.1) activities using Bio-diagnostic kit.

2.19. Statistical analysis

The analysis was performed with one way analysis of variance (ANOVA), followed by Dennett's *post hoc* test. Values of $P < 0.01$

Table 1
Total phenolic and flavonoid contents of RMAE.

Sample	Total phenolics mg GAE/100 g dry weight	Total flavonoids mg CE/100 g dry weight
RMAE	3,300 ± 120	800 ± 112

GAE, gallic acid equivalent, CE, catchin equivalent values are presented as means ± SD (n = 3).

Table 2
HPLC analysis of phenolic compounds of RMAE.

Compounds	mg/100 g	Percentage %
p-hydroxybenzoic	7.6	1.2
Catachine	9.6	1.5
Cholorgenic	4	0.64
Caffeic	12	1.92
Syrngic	3.5	0.56
Vanillic	0.355	0.056
Ferulic	1.2	0.19
Rutin	7.5	1.2
p-Coumaric	50	8
Hisperidin	54.2	8.7
Apeginin-7-glucoside	7	1.1
Myricetin	19.2	3
Rosmarinic	400	64.5
Cinnamic	0.92	0.147
Apeginin	45.7	7.3

were considered significant. Further, the results are represented in the form of mean ± S.D.

3. Results

3.1. Total phenolic, flavonoid contents and HPLC analysis of the RMAE

The total phenolic and flavonoid contents of rosemary aqueous extract (RMAE) were found to be 3,300 mg GAE/100 g and 800 mg CE/100 g, respectively, Table 1. The compounds of RMAE were identified and quantified by HPLC technique and presented in Table 2. The results indicated that the rosmarinic acid (RA) is the major compound of the RMAE (Fig. 1 B). RA represented 400 mg/100 g dry weight and 64 % of all the identified compounds, whereas the minor components were hisperidin, *p*-coumaric acid, apeginin, myrcetin, caffeic acid, catachine, *p*-hydroxybenzoic acid, rutin, apeginin-7-glucoside, cholorgenic acid, syrngic acid, ferulic acid, cinnamic acid and vanillic acid. The results indicated that the most of the RMAE components were phenolic acids.

3.2. Protease inhibition

The RMAE inhibited the Cc venom protease activity dose dependently. The extract could inhibit 50% (IC_{50}) and 100% of the venom protease activity at venom: RMAE ratios 1:4 and 1:10 (w/w), respectively (Fig. 2A). Furthermore, the inhibition of protease was proven by the zymogram analysis in Fig. 2B, where high protease activity of Cc venom on gelatin was shown as clear zone in lane 1. The RMAE reduced the clear zones of activity dose dependently and the complete inhibition of protease activity was achieved at venom: RMAE ratio of 1:20.

3.3. L-amino acid oxidase (LAAO) inhibition

The RMAE abolished the LAAO activity of the Cc venom dose-dependently. The IC_{50} value was determined at venom: RMAE (w/w) ratio of 1:2 ratio. While, the 100% inhibition achieved at 1:10 ratio (Fig. 3).

3.4. Phospholipases (PLA_2s) inhibition

The RMAE could inhibit the Cc venom PLA_2s activity dose-dependently using egg yolk suspension as lecithin source. The IC_{50} and the 100 % inhibition values were calculated at ratios 1:2 and 1:4 (venom: RMAE w/w), respectively (Fig. 4A). Furthermore, 200 μg of Cc venom caused a clear zone diameter of 0.8 ± 0.05 cm on the prepared gel. Fig. 4B showed decreasing of halos diameters by increasing the RMAE concentration, where 85% inhibition was achieved at ratio 1:5.

3.5. Hemolytic inhibition

The RMAE significantly inhibited Cc venom hemolytic activity.

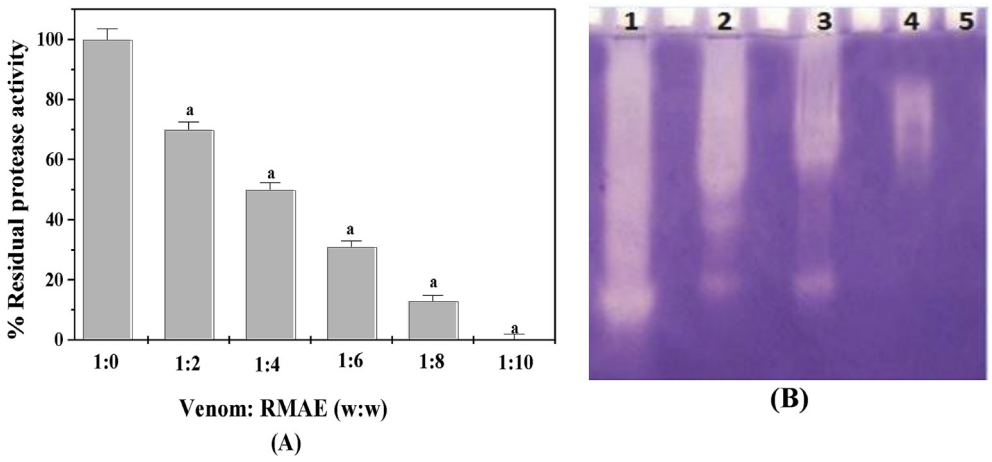

Fig. 2. (A) Protease inhibition of the of Cc venom. Five caseinolytic units (5 μg) of Cc venom pre-incubated with different of RMAE ratios (1:0, 1:2, 1:4, 1:6, 1:8 and 1:10) for 30 min at 37 °C and the residual activity was measured using standard assay. The values represent mean ± S.D. (n = 3) and the results $P < 0.01$ ([a]) were considered as significant. **(B)** Zymogram analysis of protease inhibition of the Cc venom. Lane 1, 30 μg of Cc venom alone, lanes 2, 3, 4 and 5, (venom: RMAE w/w) ratios of 1:2.5, 1:5, 1:10 and 1:20 respectively.

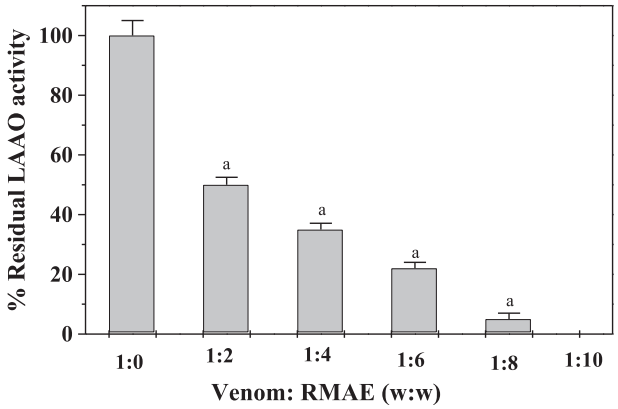

Fig. 3. L-amino acid oxidase inhibition of Cc venom. 2 μg of the Cc venom pre-incubated with different of the RMAE ratios (1:0, 1:2, 1:4, 1:6, 1:8, and 1:10) for 30 min at 37 °C and the residual activity was measured using standard assay. The values represent mean ± S.D. (n = 3) and the results $P < 0.01$ ([a]) were considered as significant.

The IC_{50} value of hemolytic inhibition was determined at ratio 1:10 (venom: RMAE w/w). While, the venom hemolytic activity was totally inhibited at ratio 1:30 (Fig. 5A). Further, hemolysis didn't appear when the RMAE incubated with the RBCs assay mixture alone. This proves that the RMAE is non-toxic.

3.6. Inhibition of coagulant activity

The procoagulant activity of Cc venom was found to be Ca^{+2} dependent as coagulation initiated after calcium chloride addition. The plasma clotting time was 40 ± 1.5 s at 1 μg Cc venom concentration, while the time was prolonged to 180 ± 8 s for the control. The RMAE prolonged the clotting time of the venom from 40 ± 1.5 s to 160 ± 7.5 s at ratio 1:20 (venom: RMAE w/w) (Fig. 5 B).

3.7. Inhibition of fibrinogen degradation

When 5 μg of Cc venom was incubated with fibrinogen for 2 h, the α and ð chains of fibrinogen were completely digested, whereas

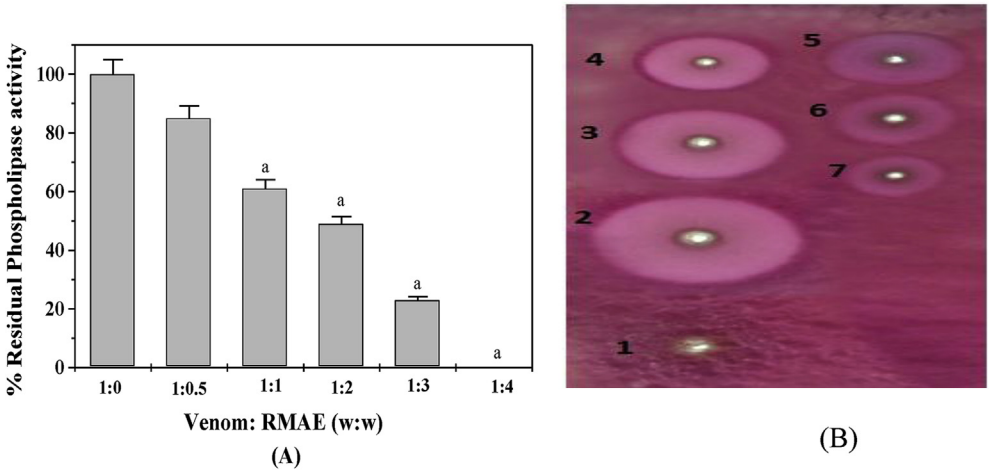

Fig. 4. (A) Phospholipases A_2 inhibition of the of Cc venom. 200 μg of the Cc venom was pre-incubated with different of the RMAE ratios (1:0, 1:0.5, 1:1, 1:2, 1:3, and 1:4) for 30 min at 37 °C and the residual activity was measured using standard assay. The values represent mean ± S.D. (n = 3) and the results $P < 0.01$ ([a]) were considered as significant. **(B)** Phospholipase A_2 inhibition of the of Cc venom in agarose gel containing 4% washed human erythrocytes, 4% egg yolk. Halo 1, RMAE alone, hole 2, 200 μg of Cc venom alone, halos 3, 4, 5, 6 and 7 (venom: RMAE w/w) ratios of 1:1, 1:2, 1:3, 1:4, and 1:5, respectively.

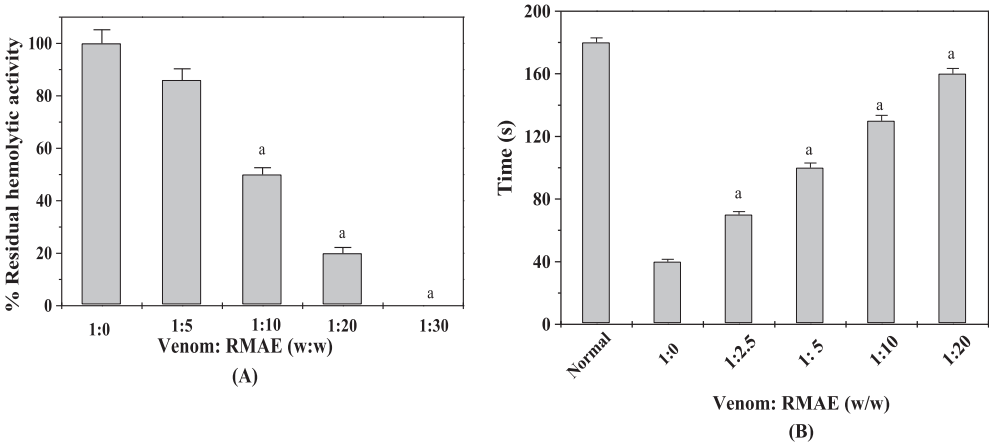

Fig. 5. (A) Hemolytic inhibition of Cc venom. 5 μg of the Cc venom was pre-incubated at different ratios of the RMAE (1:0, 1:5, 1:10, 1:20, and 1:30) for 30 min at 37 °C. **(B)** Procoagulant inhibition activity of Cc venom. 1 μg of the Cc venom was pre-incubated with different of the RMAE ratios (1:0, 1:2.5, 1:5, 1:10, and 1:20) for 30 min at 37 °C. The residual activity was measured using standard assays. The values represent mean ± S.D. (n = 3) and the results $P < 0.01$ ([a]) were considered as significant.

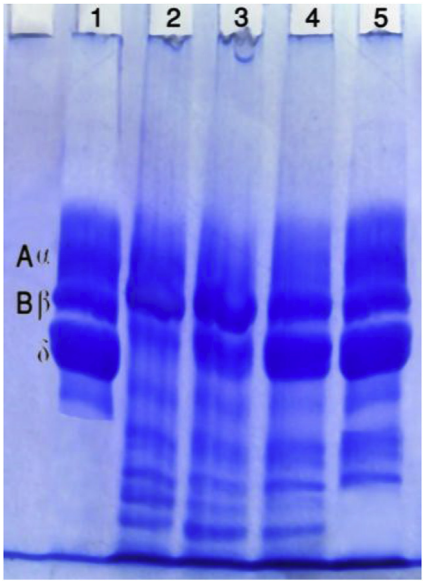

Fig. 6. Fibrinogenase inhibition activity of Cc venom. Lane 1, 2 mg/ml fibrinogen alone, lane 2, 5 μg of Cc venom + fibrinogen, lanes 3, 4, and 5 fibrinogen pre-incubated with (venom: RMAE w/w) ratios of 1:10, 1:20 and 1:40, respectively.

β-chain was partially digested. At venom/RMAE ratios 1:10 and 1:20, the preferential inhibition of β and ð chains degradation over α chain was observed, while α, β and ð chains degradation were totally inhibited at 1:40 ratio (Fig. 6).

3.8. Neutralization of Cc venom lethality

The LD_{50} of Cc venom was established at 8 μg/20 g mouse (data not shown), the i.p. administration of the RMAE didn't induce any toxic effects even after 24 h of injection. Different doses of LD_{50} of venom were injected (i.p) into 8 groups of mice with or without RMAE. The mice injected with the RMAE after 10 min of venom injection showed a significant increase in the survival time compared to the mice injected with the venom alone. Furthermore, it was observed that the RMAE complete neutralized the lethality of the 1/2 and 1 LD_{50} doses of Cc venom. About 50% of mice still survive in a group injected with (2 LD_{50} of the venom + 0.5 mg of RMAE) and the survival time was 18 h compared to 27 min for a group of mice injected with 2 LD_{50} of the venom alone. In addition, the RMAE increased the survival time in a group of mice injected with 4LD_{50} of venom up to 14 h compared to 12 min for a group of mice injected with 4 LD_{50} of the venom alone (Table 3).

Table 3
Neutralization of Cc venom lethality by RMAE administered intra-peritoneal. The values represent mean ± S.D. (n = 5). The results P < 0.01 ([a]) were considered as significant.

Groups	Number of dead mice	Survival time (h)
Group1 (1/2 LD_{50} venom alone)	1/5	23 ± 0.15
Group 5 (1/2 LD_{50} venom + 0.5 mg/ml of RMAE)	0/5	≥24
Group 2 (1LD_{50} venom alone)	3/5	20 ± 0.15
Group 6 (1LD_{50} venom + 0.5 mg/ml of RMAE)	0/5	≥24
Group 3 (2LD_{50} venom alone)	5/5	0.45 ± 0.10
Group 7 (2LD_{50} venom + 0.5 mg/ml of RMAE)	3/5	18 ± 0.91[a]
Group 4 (4LD_{50} venom alone)	5/5	0.20 ± 0.02
Group 8 (4LD_{50} venom + 0.5 mg/ml of RMAE)	5/5	14 ± 0.10[a]
Group 9 Saline	0/5	≥24
Group 10 (0.5 mg/ml of RMAE)	0/5	≥24

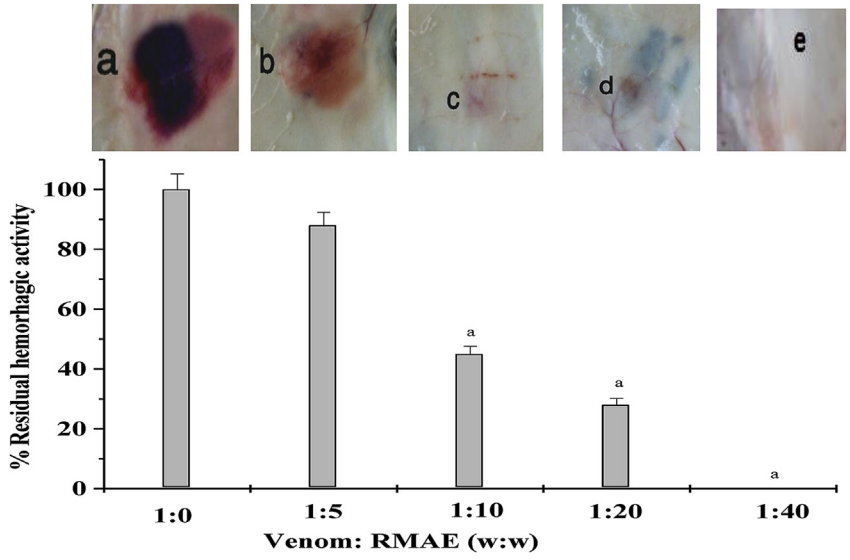

Fig. 7. Hemorrhagic inhibition of the Cc venom. The spots were developed in the skin of mice with doses of (a) 2MHD of venom alone, (b, c, d and e) 2MHD of venom pre-incubated with different ratios of the RMAE (1:5, 1:10, 1:20 and 1:40), respectively. The values represent mean ± S.D. (n = 5). The results P < 0.01 ([a]) were considered as significant.

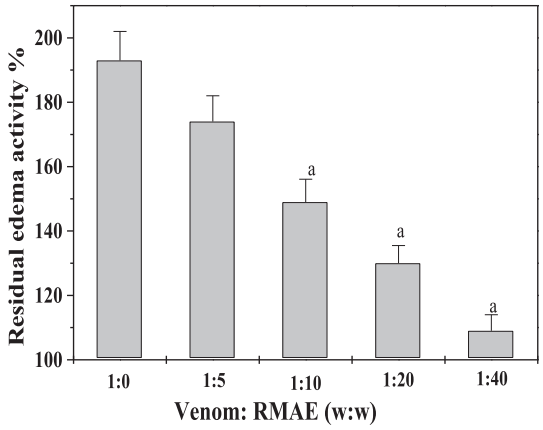

Fig. 8. Inhibition of the edema activity of Cc venom. 20 μg (5 MED) of the Cc venom was pre-incubated with different ratios of the RMAE (1:0, 1:5, 1:10, 1:20 and 1:40) for 30 min at 37 °C before injected in mice. The residual activity was measured using standard assay. The values represent mean ± S.D. (n = 5) and the results $P < 0.01$ ([a]) were considered as significant.

Table 4
Serum ALT, AST and CPK levels of mice injected with Cc venom in the absence and presence of RMAE. The values represent mean ± S.D. (n = 5) and the results $P < 0.01$ ([a]) were considered as significant.

Groups	Serum ALT	Serum AST	Serum CPK
Saline (Control)	42 ± 1.6	51 ± 2.04	57 ± 2.2
Venom alone	175 ± 5.2	182 ± 5.8	366 ± 6.1
Venom + 0.5 mg/ml of RMAE	52 ± 1.6[a]	61 ± 1. 2[a]	75 ± 1.5[a]
0.5 mg/ml of RMAE	43 ± 1.63	52 ± 1.7	62 ± 1.4

3.9. Neutralization of hemorrhagic activity of Cc venom

It was observed that the diameter of hemorrhagic spot in dorsal skin of mice injected with 1 μg of Cc venom was about 21 ± 1.4 mm. While, the diameters of hemorrhagic spots were reduced dose dependently with increasing the RMAE concentrations and the 100 % neutralization of hemorrhagic activity was achieved at ratio 1:40 (venom: RMAE w/w) (Fig. 7). Furthermore, the RMAE alone didn't cause any hemorrhage.

3.10. Neutralization of edema inducing by Cc venom

The RMAE showed a strong neutralization of edema inducing by Cc venom. When the Cc venom was incubated with RMAE and injected into the mice feet, a significant decrease in the edema ratio was observed. The RMAE reduced the edema from 193 ± 9.1% to 133 ± 5.5% at ratio 1:20 ratio (venom: RMAE w/w). Furthermore, at venom/RMAE ratio 1:40 the edema was reduced to 109 ± 4.7% (Fig. 8). The mice injected with RMAE alone showed an edema of 105 ± 4.9% which represents further evidence of non-toxicity of RMAE in mice models.

3.11. Neutralization of myotoxicity of Cc venom

The Cc venom induced pronounced myotoxicity, which was measured by increasing in the serum CPK level (Table 4). Whereas, the CPK level was highly decreased and 84.4% of the venom myotoxic was inhibited in mice groups injected with 0.5 mg/ml RMAE after 10 min of the venom injection. Moreover, no increasing in enzymatic activity recorded when a group of mice injected individually with RMAE alone. In addition, Fig. 9 showed the light microscopic observation of non-treated and treated mice muscle tissue sections. Hemorrhage, destructions of muscle cells and inflammatory infiltration were the prominent characteristics of muscle of envenomed mice compared with control mice. The hemorrhage and inflammation decreased and the muscle fibers appeared to be normal in a group of mice treated with RMAE.

3.12. Histopathological studies

Histopathological analysis of the liver of mice injected with Cc venom in the presence and absence of the RMAE was performed (Fig. 10). The mice injected with Cc venom alone showed evident alterations in the liver. In contrast, the used of RMAE significantly reduced the venom toxicity in liver. Histological investigation of hepatic tissue sections revealed that Cc venom caused a severe liver inflammatory response, as indicated by inflammatory cellular infiltration as well as cytoplasmic vacuolation and degeneration of hepatocytes. Further, scattered dilated, congested central and portal blood vessels were observed and the hepatic sinusoids were dilated and apparently contained more Kupffer cells. Normal histological features of the liver of mice treated with RMAE. In addition, an obvious increase in AST and ALT enzymatic activities was

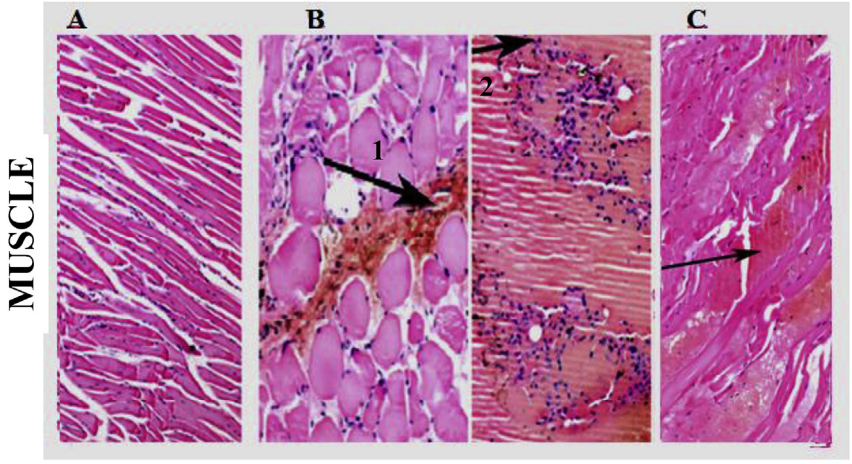

Fig. 9. Effect of saline (A), lethal dose of Cc venom (B) and RMAE (C) on the site of injection of mice muscle. Photomicrographs in (A) showed normal skeletal muscle fibers, (B) showed hemorrhage (thick arrow1) and inflammation (thick arrow2) with degenerated muscle fibers and in (C) showed normal appearance of muscle fibers with less hemorrhage (thin arrow). Sections were stained with hematoxylin-eosin dye (H&E X 160).

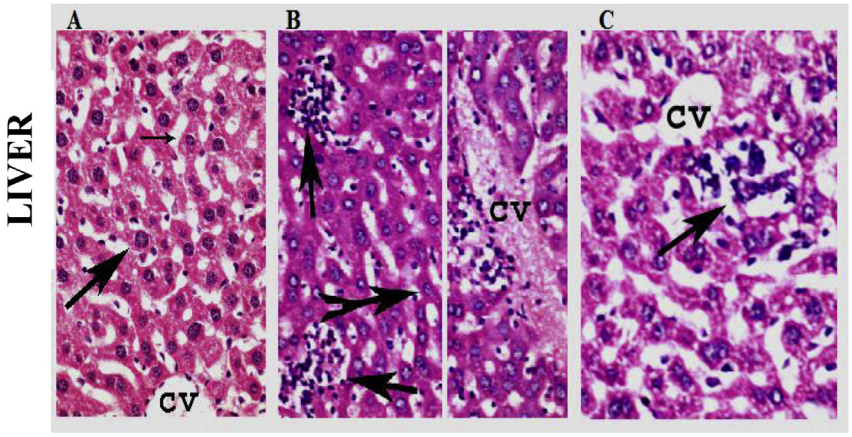

Fig. 10. Histological examination of mice liver. (A) a normal architecture of liver tissue with normal hepatocytes (thick arrow), central vein (CV) and thin sinusoid (thin arrow), (B) a marked dilated and congested central vein (CV), shrieked hepatocytes (arrow with 2 tail) and aggregated inflammatory cells (thick arrow) were seen in mice liver injected with Cc venom alone, (C) liver section of mice treated with RMAE showed normal central vein (CV) with few necrotic cells (thick arrow). Sections were stained with hematoxylin-eosin dye (H&E X 400).

recorded in groups of mice injected with Cc venom alone. On the other hand, an obvious decrease in AST and ALT enzymatic activities, almost close to negative control levels, was recorded in mice injected with RMAE after venom injection, Table 4.

4. Discussion

Although antivenom is the only available specific treatment, it doesn't provide enough protection against venom. Further, it stimulates local tissue injury and often develops hypersensitivity reactions.[33] Searching for safe and effective anti-venom compounds from natural sources is very important. Rosemary leaves extracts were investigated as therapeutic potential agents against several diseases.[7–13]

In the present study, rosemary leaves aqueous extract (RMAE) contained a considerable amount of phenolic and flavonoid contents. Difference of total phenolic and flavonoid contents of rosemary extracts were recorded in previous studies.[34,35] These differences might be due to rosemary cultivars, geographical location, and extraction conditions. Further, a considerable variation was found in phenolic acids of RMAE by using HPLC. Some of them were similar to those reported previously.[35–37] Many studies reported that rosmarinic acid (RA) was the major phenolic compound in rosemary extracts. Our results recorded that the concentration of RA was much higher than that reported previously.[34,35,38] Moreover, previous studies proved that the rosemary extracts and their constituents had antioxidant and anti-inflammatory activities.[34,39–42] The inhibitory potential of RMAE against Cc crude venom was evaluated by two strategies, the first one based on pre-incubation of venom with RMAE for a time prior determining the residual activities of the viper enzymes (in vitro assay) and the second involved administration of the extract after venom injection (in vivo assay).

The proteome wealth of Cc viper venom responsible for the hemostatic disorders and local tissue injury observed after viper envenomation. The Cc venom contains 70% metallo, serine and hemorragic proteinases.[3,43] These proteases caused local hemorrhage, fibrinolysis, and interfere with clotting factors and the complement system.[44] The RMAE effectively inhibited the Cc venom proteases and almost the hemostatic alterations induced by Cc venom like hemorrhage, RBCs lysis, coagulation and fibrinogen degradation. The RA is an abundant phenolic component in RMAE, it has been proven to inhibit the proteinase and hemorrhagic activities of the *T. flavoviridis, C. atrox, G. blomhoffii, B. arietans* crude venoms.[45]

PLA_2s represent 19 % of Cc venom and are well known for their necrotic and inflammatory actions. The PLA_2s necrotic action involves the disruption of cell membrane and generates inflammatory mediators.[3] The RMAE effectively inhibited the crude venom PLA_2s. This inhibition may be due to the anti-inflammatory action of RMAE and its components. The RA of *Rosmarinus officinalis* has been shown to decrease the production of the inflammatory mediators resulting in stopping of the inflammation process.[8] Also, RA of *Cordia verbenacea* inhibited the main PLA_2 of *B. jararacussu* venom, which modeled into the hydrophobic channel of the enzyme active site.[46]

Egyptian Cc viper represents the richest source of LAAOs among all vipers inhabiting in the Egyptian environment.[47] LAAOs are associated with apoptotic, cytotoxic, platelet aggregation, edema and hemorrhage due to excess production of H_2O_2.[48] RMAE completely inhibited the Cc venom LAAOs activity. This inhibition may be due to the antioxidant action of RMAE and its components. Rosemary extract had higher antioxidant activity because it contained potential natural antioxidants such as RA, caffeic, ferulic, p-coumaric and p-hydroxybenzoic acids.[39,41,42] Moreover, the most antioxidant activity could be seen in the extracts with more RA.[40]

Edema is also remarkable local effect of the Cc venom envenomation.[49] The collective action of venom enzymes as (PLA_2s and LAAOs) and release of histamine from the mast cells resulting in edema.[50] RMAE considerably protected the mice feet from edema induced by the venom. This may be due to the ability of RMAE to inhibit the venom PLA2s and LAAOs and prevent releasing of histamine from the mast cells. Al-Sereiti et al.[8] reported that the rosemary extract had a therapeutic potential in treatment bronchial asthma by inhibiting the histamine action. Also, RA of *Cordia verbenacea* neutralized the edema induced by *B. jararacussu* venom.[46]

The lethality of the Cc venom effectively reduced after the injection of RMAE. Since the mice injected with lethal dose of the venom were still alive even after 24 h and the survival time was highly increased in groups of mice injected with $2LD_{50}$ and $4LD_{50}$ of the Cc venom. This was supported by the histological examinations where the RMAE improved the severe damage and inflammatory response occurred in mice liver and muscle after Cc venom injection. Mors et al.[6] reported that the RA inhibited the lethal action of the *B. jararaca* venom in mice. Wahby et al.[14] proved that the RMAE, a potent hyaluronidase inhibitor, inhibited the spreading property

of the Cc venom.

Clinically CPK assayed in blood tests is an enzymatic index of cellular damage. A remarkable decrease of' CPK levels of the envenomed mice serum confirmed that the RMAE could effectively inhibit the cellular damage (myonecrosis) induced by Cc venom. Ticli et al.[46] evidenced the anti-myotoxic properties of RA against the basic PLA_2s of *B. jararacussu* venom. Moreover, high levels of ALT and AST signifying the hepatotoxicity, a remarkable decrease of AST and ALT levels confirmed that the RMAE relieved the mice liver damage induced by the venom. The relief of liver toxicity is one of the therapeutic potentials of rosemary extract.[8]

5. Conclusion

In the present study, rosemary aqueous extract (RMAE) contained a considerable amount of phenolics, falvonoids and variation of the phenolic acids. Rosmarinic acid (RA) is the major compound of the RMAE and may be responsible for the potential neutralization action against proteases, fibrinogenases, $PLAs_2$, LAAOs, hemolytic, hemorrhagic, edema, myotoxic activities and lethality of the Egyptian *Cerastes cerastes* viper venom. The RMAE is considered as a promising remedy for lethal viper bites.

Author contributions

W. H. Salama, A. M. Abdel-Aty and A. S. Fahmy had the original idea for the study and carried out the design, **W. H. Salama and A. M. Abdel-Aty** were responsible for data analysis and data cleaning. Drafted the manuscript was revised by all authors. All authors read and approved the final manuscript.

Conflict of interest statement

All authors work in the same institute and no conflict of interest.

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