IN VITRO CYTOTOXICITY INDUCED BY THE SCORPION VENOM OF SCORPIO MAURUSPALMATUS INHABITING DIFFERENT LOCALITIES IN EGYPT.

BY

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ABSTRACT

Background: Scorpion mauruspalmatus belongs to the family scor-pionidae and is common in the Saudi Arabia, Middle East, and Mediterranean and Jordan regions. It is found in Lower Egypt (Wadi Natrun, Cairo, Faiyum, Western Mediterranean costal Desert), southern and central Sini. **Study aim**: is to study the cytotoxicity induced by the scorpion venom of Scorpio mauruspalmatus inhabiting different localities in Egypt using different in vitro approaches. **Material and methods:** Collection of scorpions and preparation of venom samples. Captive scorpions from the four locations (Alexandria, Rahaba, Sahab, and Alagramia) were kept separately in individual containers. Scorpions were milked using the squeezing method, and individual venom samples collected and lyophilized. The freeze-dried pooled venom was stored at -20 °C prior to use. **Study design**: To study in vitro cytotoxicity of *S. m. palmatus* venom collected from different locations in Egypt,blood samples were collected from 20 ether-anesthetized mice using periorbital puncture. Heparinized blood was divided into 10 groups and incubated with two different concentrations (10 and 20 ug/ml) of *S. m. palmatus* venom, and a range of assays. Assays: Malondialehyde (MDA) assay, Protein carbonyl assay, Estimation of nitric oxide, Estimation of reduced glutathione (GSH), Cu/Zn-superoxide dismutase and catalase, Estimation of hemoglobin contentwere carried out after 0, 30,60, 90 and 120 min. **Results**: The haemoglobin level was significantly ($p<0.05$) increased with time as revealed by ANOVA. According to Two-way analysis of variance there were a highly significant difference in glutathione level induced by groups ($p<0.001$***), sites ($p<0.001$***), concentrations ($p<0.001$***). The MDA level was increased in both Alexandria and Rahaba, at concentration of 20ug/ml. The protein carbonyl (PC) level in Agramia was increased at both 10,20ug/ml concentration. Also, the nitrocoxide(NO) was increased at both Alexandria and Sahab at 10ug/ml and also remarkably increased in Alexandria and Rahaba at 20ug/ml. In addition to that, the catalase level was also increased in Sahab site ato 20ug/ml concentration. The glutathione level (µg/ mL) was significantly ($p<0.05$) increased with time as revealed by ANOVA in Alexandria, Rahaba, Agramia, however it was decreased in Sahab. According to Two-way analysis of variance there were a highly significant difference in SOD Activities induced by groups ($p<0.001$***), sites ($p<0.001$***), concentrations ($p<0.001$***).

Conclusion: The venom of scorpion mauruspalmatus has significant cytotoxic effects and increase the biomarker stress, especially at 20ug/ml on different location of the study.

Keywords: *Scorpion venom toxicity, Hb, MDA, GSH, Cu/Zn-superoxide dismutase, and catalase*

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INTRODUCTION

Scorpion bits cause severe worldwide health problems in several areas of the world, not only because of their high incidence in well known areas but also because of their power to induce highly disquieting manifestation, sometimes with lethaleffectparticularly in children.The severity of scorpion toxicity depends on scorpion species, structure of the venom , and the victims' age. Furthermore, health services are hardly able to supply an sufficient therapy, as remedy of scorpionism is compound ***(BenAbdalrezek et al., 2022).***

Scorpions, poisonous arthropods, correlate to Arthropoda phylum, Arachnidacategory and scorpions order which are classified in 16 groups and 1500 species and subspecies in the world ***(Chowell et al., 2006).*** Scorpion venom encompasses mucopolysacharides, hya-luronidase, phospholipase, serotonin, histamine, enzyme inhibitors and neurotoxic peptides**.** Scorpion mauruspalmatus belongs to the family scor-pionidae and is widespread in the, Middle East, and Mediterranean regions ***(Abdel-Rahman et al., 2009)***.

With the advance in research and expansion and growth inscience and technology, new approaches have been espoused to disclose

venom toxins that do not linkantivenoms but could integrate to form venom vaccination admixtures ***(Casewell et al., 2013).***

The impact of venoms can be the fundamental for research and advancement of remedy protocols essential in the modification of the immunological system, including diverse autoimmune disorders ***(Avalo et al., 2022).***

Recently, the field of peptide based drug discovery has seen a breakthrough where in hundreds of academic research groups as well as big drug design firms are permanently working on it. During 2015–2019, 208 new drugs, of which 150 are new chemical entities and 58 are biologics have been confirmed by United States, food and drug administration ***(Suhas, 2020).***

Cytotoxicity of scorpion venom that are thought to be behind the pathological and clinical manifestations of envenoming. With the advent of both molecular biology and biotechnological approaches a widerange of bioactive molecules can be found, separated, and studied in scorpion venoms. Also, effective vaccines could be developed and applied in Emergency Medicine and clinical Toxicology. In addition, some scorpion venom peptides might have pharmacologic properties which could be investigated as therapeutic agents against some chronic and refractory disorders, especially in disorders with drug resistance ***(Soopramanien, et al., 2020).***

Most victims of scorpion stings suffer severe pain, but a few especially children, develop system envenoming which is dominated by cardiovascular and respiratory consequences of hypercatecholminic myocardites. Other clinical manifestations include paralysis. Coagulapathy and local tissue injury, bleeding and acute injury ***(Amret al., 2021).***

The pathogenesis of cardiac dysfunction and myocardial damage secondary to scorpion envenomation had largely been the subject of debate in the past. The most accepted hypothesis was the increased catecholamine circulating secondary to a direct stimulatory effect of the venom on the adrenals and on sympathetic nerve endings. This hypothesis was confirmed by some clinical and experimental studies ***(Zeghal et al., 2000).***

Scorpion mautuspalmtaus have been selected for our study because of it is common and widely distributed in Egypt particularly in both arid and semiarid habitats. In addition, it has a unique venom structure, as well as its features and assumed therapeutic effects.

AIM OF THE WORK

is to study the cytotoxicity induced by the scorpion venom of Scorpio mauruspalmatus inhabiting different localities in Egypt using different in vitro approaches.

MATERIALS AND METHODS

- *Collection of scorpions and preparation of venom samples:*

Samples of S. m. palmatus were collected from two different geographical regions in Egypt, the Sinai Peninsula and the North coast. Scorpions were captured from three different locations (WadiSahab, El-Agramia and Rahaba Plains) in the southern region of the Sinai Peninsula (910–1676 m above sea level), an area geographically separated from the Western Mediterranean Coastal Desert (30.5 m above sea level) by the Suez Canal and Gulf of Suez from where a second group of scorpions were collected ***(Abdel-Rahman et al., 2009).*** Captive scorpions from the four locations were kept separately in individual containers. Scorpions were milked using the squeezing method according to ***(Abdel-Rahman et al., 2009)*** and individual venom samples collected and lyophilized. The freeze-dried pooled venom was stored at -20 °C prior to use.

- *Study design:*

To investigate the cytotoxicity of S. m palmatus in vitro, venom gathered from several locations in Egypt, blood samples were gathered from 20 ether-anesthetized mice using periorbital puncture. Heparinized blood was divided into 10 groups and incubated with two different concentrations (10 and 20 ug/ml) of *S. m. palmatus* venom and a range of assays (malonaldehyde, protein carbonyl content, nitric oxide, reduced glutathione, Cu/Zn-superoxide dismutase, catalase and *hemoglobin content*) were carried out after 0, 30, 60, 90 and 120 min.

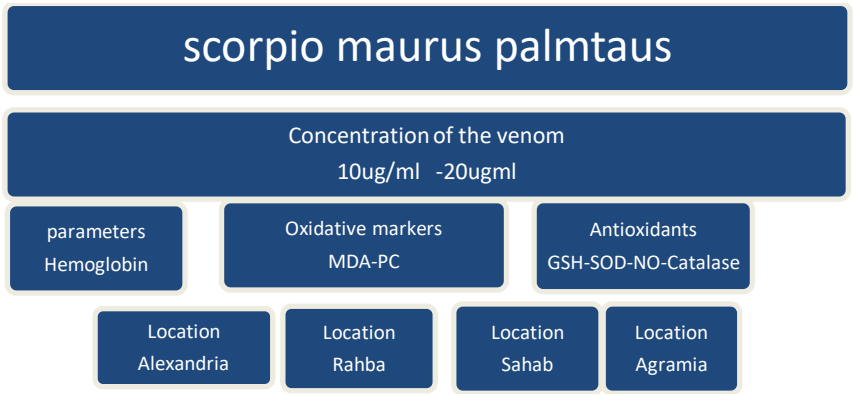

Fig. (1): ***Flow diagram shows the scorpion venom concentration, parameters, and locations***

- *Malonaldehyde (MDA) assay:*

Plasma MDA formation was used to quantify the lipid peroxidation level in the *whole mouse blood*. It was measured using the thiobarbituric acid (TBA) assay according to the method of ***Uchiyama and Mihara (1978)*** by reacting of 0.5 ml of a 10% (w/v) blood with 1 ml of 6% thiobarbituric acid (Win lab, UK) and 3 ml of 1% phosphoric acid for 45 min in boiling water. The color of TBA chromogen was measured at 520 and 532 nm (Helios a UNICAM UV-Visible Spectrophotometer, UNICAM, Cambridge, UK). The difference between absorbance at 520 nm and at 530 nm is used to determine the TBA value which represents the malonaldialehyde concentration and was taken as the measure of lipid peroxidation in blood. The compound 1,1,3,3-tetramethoxypropane was used as an external standard.

- *2.2.2. Protein carbonyl assay:*

Plasma protein carbonyl content (PCC), as a marker of protein oxidation, was measured according to **Reznick and Packer (1994).** Protein was precipitated with an equal volume of 1% trichloroacetic acid (TCA) and the pellet was resuspended in 1 mL of 2,4-dinitrophenylhydrazide (DNPH (Sigma), 10 mM, dissolved in 2 N HCl). Samples were left at room temperature for 1 h in the dark and vortexed every 15 min. An equal volume of 20% TCA was added and after centrifugation (12,000 g, 1 min, 4 °C), pellets were washed three times with 1 mL of an ethanol: ethyl acetate mixture (1:1) to remove the free DNPH. The final pellet was dissolved in 1 mL of 6 M guanidine and kept at 37 °C for 1 h in a shaking water bath. The solution was centrifuged (12,000 g, 15 min) and the carbonyl content (nmol/mg), measured as protein phenylhydrazone derivatives, was determined at 370 nm using an absorption coefficient of 22,000 $M^{-1}C\ m^{-1}$.

- *Estimation of nitric oxide:*

Nitric oxide (NO) was measured using the Griess reaction **(Green et al., 1982)**. Plasma (100 µL) was mixed with an equal volume of Griess reagent (1 part 0.1% naphthyl-ethylene diamine-dihydrochloride in distilled water plus 1 part 1% sulfanilamide in 5% concentrated H_3PO_4), at room temperature for 10 min. The absorbance was measured at 540 nm. Sodium nitrite was used as a standard.

2.2.4. Estimation of reduced glutathione (GSH)

The content of blood glutathione of control and treated groups was estimated according to the method **of *Beutler et al. (1963).*** Aliquots of 0.2 mL of blood were added to 1.8 mL distilled water and 3 mL of precipitating solution (1.67 g glacial metaphosphoric acid, 0.2 g EDTA and 30 g NaCl in 100 mL distilled water). The mixture was then centrifuged (2200 g, 15 min, 4 °C). One milliliter of supernatant was added to 4 mL Na 2 HPO_4 (0.3 M) and 0.5 mL dithiobis-2-nitrobenzoic acid reagent (DTNB, Sigma-Aldrich) (40 mg DTNB in 100 mL 1% sodium citrate) and the absorbance was measured at 412 nm. Glutathione reduced form (Sigma) was used as a standard.

2.2.5. Cu/Zn-superoxide dismutase and catalase

Superoxide dismutase (Cu/Zn SOD; EC 1.15.1.1) activity was estimated in red blood cell hemolysate according to ***Misra and Firdovich (1972).*** The rate of inhibition of auto oxidation was monitored at 560 nm; the amount of enzyme required to produce 50%

inhibition is defined as one unit of enzyme activity. Cu/Zn SOD activity was expressed as units/mL. Catalase (CAT; EC 1.11.1.6) activity was assayed using the method of ***Aebi (1984)***, following the decomposition of H_2O_2 at 240 nm. Activity was expressed as units/mL.

2.2.6. Estimation of hemoglobin content

Hemolytic activity of the crude venom (10 and 20 ug/ml) from all scorpion populations were evaluated using the whole blood of mice at different time intervals (0, 30, 60, 90 and 120 min) through measuring the level of released Hemoglobin ***(Corzo et al., 2001)***. Hemoglobin content in both control and treated groups was estimated using hemoglobin colorimetric detection kit obtained from Biomerieux Company (Marcy l'Etoile, France).

STATISTICAL ANALYSIS:

The statistical analysis will be performed for To assess and compare between Control and Treated (A_0, A_1) groups at different time points (T_0, T_{30}, T_{60}, T_{90}, T_{120}) at four different sites (S1=Alexandria, S2= Rahaba, S3=Agramia, S4=Sahab); repeated measures MANOVA is proposed (ANOVA).The data will be collected, checked, revised, and organized in tables and figures using Microsoft Excel 2016. Data will be subjected to outliers' detections and normality statistical test to detect whether the data are parametric or nonparametric.

Data will be analyzed for descriptive statistically both graphical and numerical description. Inferential statistics for evaluating and comparing groups, time points, concentrations, and their interactions will be performed by repeated measures analysis of variance (ANOVA) or corresponding nonparametric analyses at significance levels of 0.05. ANOVA will be followed by Duncan multiple range tests (DMRTs) to compare between treatment groups. Data analyses will be carried out using computer software Statistical Package for Social Science SPSS (IBM-SPSS ver. 23.0 for Mac OS) ***(Knapp, 2017).*** The principal component analysis (PCA) will be carried out, it is a technique for reducing the dimensionality of such data sets, increasing the interpretability but at the same time minimizing information loss.

Sample size calculations

To assess and compare between Control and Treated (A_0, A_1) groups at different timepoints (T_0, T_{30}, T_{60},T_{90},T_{120}) at four different sites (S1=Alexandria, S2= Rahaba, S3=Agramia, S4=Sahab); repeated measures MANOVA is proposed (ANOVA). A minimum total sample size of 80 samples will be sufficient to detect the effect size of 0.25, a power (1-β=0.90) of 90% at a significance probability level of $p<0.05$ partial eta squared of 0.06. According to sample size calculations treatment group (A_0, A_1), and time of investigations (T_0, T_{30}, T_{60}, T_{90}, T_{120}) would be represented by a minimum of 5 samples as shown in Tables 1 and 2. The sample size was calculated according to G*Power software version 3.1.9.6 ***(Faul et al., 2007).***

Where;*f:* is the effect size; α= 0.05; β=0.10; Power= 1- β = 0.90

$$f = \frac{\sigma_\mu}{\sigma}$$

$$\sigma_\mu^2 = \frac{\sum_{i=1}^{k} n_j (\mu_i - \mu)^2}{N}$$

RESULTS

1- **Haemoglobin level:** The haemoglobin level measured at different study sites (Alexandria, Rahaba, Agramia, and Sahab) at different concentration (10, 20 µg/mL), treatment groups (Control, Treated) at different timepoints (0, 30, 60, 90, 120) were presented in table (1) .

The haemoglobin level was significantly ($p<0.05$) increased with time as revealed by ANOVA. Alexandria site showed a haemoglobin level of 13.6, 13.3., 14.4, 14.2, and 14.7g/dL at 0, 30, 60, 90, 120 minutes at control group, however, it recorded a haemoglobin level of 12.5, 13.8, 13.5, 13.3, and 14.8g/dL in the treated group of 10 µg/mL. Differences between subgroups was assessed using Duncan's Multiple Range test. Means followed by different letters are significantly different according to DMRTs at 0.05 level.

The haemoglobin in all studied sites and groups ranged between 7.16g/dL recorded 90 minutes in the control group of Alexandria, to a highest level of 18.40g/dL recorded at level

highest haemoglobin level was recorded at Agramia site treated group both at 10 µg/mL.
According to Two-way analysis of variance there were a highly significant difference in haemoglobin level induced by groups (p<0.001***), sites (p<0.0001***), concentrations (p<0.001***). Moreover, the interaction between groups and sites showed a highly significant difference (Table 7, MANOVA table). Furthermore, the Time also induced a highly significant differences in haemoglobin level. A significant increase in haemoglobin level with dose 10 to 20 µg/mL was noticed at both control and treated groups at each time points as revealed by DMRTs at 0.05 level.

Table (1): Haemoglobin level at different study sites (Alexandria, Rahaba, Agramia, and Sahab) at different concentration (10, 20 µg/mL), treatment groups (Control, Treated) at different timepoints (0, 30, 60, 90, 120)

Site	Conc. (µg/mL)	G	Hb at different time points				
Alexandria			**T0**	**T30**	**T60**	**T90**	**T120**
		C	7.8	7.7	7.7	7.2	7.6
	10	T	7.8	7.7	7.7	7.2	7.6
		C	15.0	12.5	12.0	12.5	12.1
	20	T	14.1	12.0	12.0	12.1	12.7
Rahaba		C	13.6	13.3	14.4	14.2	14.7
	10	T	12.5	13.8	13.5	13.3	14.8
		C	11.5	12.7	11.6	12.9	12.8
	20	T	12.8	12.8	13.2	12.4	13.9
Agramia		C	12.3	13.0	11.5	12.5	16.1
	10	T	13.7	15.1	15.2	13.6	18.4
		C	11.7	11.4	12.0	11.0	15.9
	20	T	12.4	13.2	12.8	13.2	17.7
Sahab		C	11.3	11.6	10.5	9.4	8.7
	10	T	14.1	12.7	12.7	15.6	12.0
		C	10.0	9.9	9.5	8.8	10.6
	20	T	12.1	12.7	13.9	12.4	10.8
ANOVA (p-value)			>0.05 ns	<0.001***	<0.001***	<0.001***	<0.001***

**** significant at p<0.05, <0.01, <0.001, NS, nonsignificant at p>0.05.*

2- GSH

The glutathione level (µg/ mL) estimated at different study sites (Alexandria, Rahaba, Agramia, and Sahab) at different concentration (10, 20 µg/mL), treatment groups (Control, Treated) at different timepoints (0, 30, 60, 90, 120) were presented in table (2).
The glutathione level (µg/ mL) was significantly (p<0.05) increased with time as revealed by ANOVA in Alexandria, Rahaba, Agramia, however it was decreased in Sahab. Alexandria site showed a glutathione level (µg/ mL) of 1,787.2, 1,695, 1,836, 1,555, 1,439 µg/ mL at 0, 30, 60, 90, 120 minutes at control group, however, it recorded a glutathione level of1,843.1,659.2, 1,806, 1,712.6, and 1,542µg/mL in the treated group of 10 µg/mL. Differences between subgroups was assessed using Duncan's Multiple Range test. Means followed by different letters are significantly different according to DMRTs at 0.05 level.
The glutathione in all studied sites and groups ranged between 282.0±56.4 µg/ mL recorded T0 in the control group of Alexandria (20 µg/mL), to a highest level of 2239.8±185 µg/ mL recorded at level highest glutathione level was recorded at Agramia site treated group at 10 µg/mL.
According to Two-way analysis of variance there were a highly significant difference in glutathione level induced by groups (p<0.001***), sites (p<0.001***), concentrations (p<0.001***).

Table (2): The glutathione level (µg/ mL) estimated at different study sites (Alexandria, Rahaba, Agramia, and Sahab) at different concentration (10, 20 µg/mL), treatment groups (Control, Treated) at different timepoints (0, 30, 60, 90, 120)

Site	Conc. (µg/mL)	G	GSH at different time point					2 way ANOVA		
Alexandria			**T0**	**T30**	**T60**	**T90**	**T120**			
	10	C	1,7	1,6	1,8	1,5	1,4			
		T	1,84	1,6	1,80	1,71	1,54	<0.001	>0.05	<0.001
	20	C	1,41	1,64	1,48	1,3	1,2			
		T	1,6	1,9	1,8	1,7	1,7			
Rahaba	10	C	1,2	1,4	1,49	1,34	1,35			
		T	1,33	1,34	1,34	1,43	2,01			
	20	C	1,33	1,42	1,50	1,74	1,54	0.008	>0.05	<0.001
		T	1,16	1,24	1,32	1,93	1,75			
Agramia	10	C	1,57	1,6	1,9	1,79	1,59			
		T	1,71	2,23	1,8	1,65	1,7			
	20	C	1,5	1,89	1,59	1,4	1,58			
		T	1,6	1,9	1,6	1,48	1,6	0.008	>0.05	0.012
Sahab	10	C	1,78	1,69	1,8	1,55	1,4			
		T	1,8	1,65	1,8	1,7	1,5			
	20	C	1,4	1,6	1,48	1,36	1,28			
		T	1,69	1,9	1,8	1,7	1,7	0.012	>0.05	<0.001

Significant at p<0.05, <0.01, <0.001, NS, nonsignificant at p>0.05.

3- SOD

The superoxide dismutase activity (SOD, U/mL) estimated at different study sites (Alexandria, Rahaba, Agramia, and Sahab) at different concentration (10, 20 µg/mL), treatment groups (Control, Treated) were presented in table (3).

The SOD activity was significantly (p<0.05) increased in treated group as compared to control group as revealed by ANOVA in Alexandria, Rahaba, Agramia, Sahab. The SOD activity under 10 µg/mL in control group in Alexandria, Rahaba, Agramia, Sahab showed an average (±SD) of 350.86±10.15, 160.80±5.12, 311.80±11.32, and 236.00±13.17, however, treated groups showed a higher level recorded an average(±SD) 156.86±8.63, 132.60±17.73, 193.20±6.98, and 72.20±27.11. The difference between groups was assessed using DMRTs at 0.05 level, where means with different letters are significantly different.

Furthermore, the SOD activity under 20 µg/mL in control group in Alexandria, Rahaba, Agramia, Sahab showed an average (±SD) of 225.20±7.32, 170.40±2.99, 157.20±4.87, and 135.60±15.29, however, treated groups showed a higher level recorded an average (±SD) of 279.40±5.50, 228.80±3.61, 211.60±2.30, and 273.80±7.16; respectively.

According to Two-way analysis of variance there were a highly significant difference in SODA ctivities induced by groups (p<0.001***), sites (p<0.001***), concentrations (p<0.001***). Moreover, the interaction between groups and sites showed a highly significant difference.

Table (3): The superoxide dismutase activity (SOD, U/mL) estimated at different study sites (Alexandria, Rahaba, Agramia, and Sahab) at different concentration (10, 20 µg/mL), treatment groups (Control, Treated).

Conc. (µg/mL)	G	SOD activity (Mean ± SD)			
		Alexandria	Rahaba	Agramia	Sahab
10	C	350.9±10.2	160.8±5.1	311.8±11.3	236.0±13.2
	T	156.9±8.6	132.6±17.7	193.2±7.0	72.2±27.1
20	C	225.2±7.3	170.4±3.0	157.2±4.9	135.6±15.3
	T	279.4±5.5	228.8±3.6	211.6±2.3	273.8±7.2
Univariate analysis					
Corr.model		<0.001 ***	Site x Group		<0.001 ***
Group		<0.001 ***	Site x conc.		<0.001 ***
Conc.		<0.001 ***	Group x conc.		<0.001 ***
Site		<0.001 ***	Site x Group x conc.		<0.001 ***

*, **, *** *significant at p<0.05, <0.01, <0.001, NS, nonsignificant at p>0.05.*

4- Catalase activities

The catalase activities (CAT, U/mL) estimated at different study sites (Alexandria, Rahaba, Agramia, and Sahab) at different concentration (10, 20 µg/mL), treatment groups (Control, Treated) were presented in table (4).

The Catalase activity was significantly (p<0.05) increased in treated group as compared to control group as revealed by ANOVA in Alexandria, Rahaba, Agramia, Sahab. The catalase activity under 10 µg/mL in control group in Alexandria, Rahaba, Agramia, Sahab showed an average (±SD) of 0.23±0.02, 0.41±0.00, 0.51±0.01, and 0.59±0.01, however, treated groups showed a higher level recorded an average (±SD) 0.33±0.03, 0.42±0.02, 0.55±0.01, and 0.59±0.01. The difference between groups was assessed using DMRTs at 0.05 level, where means with different letters are significantly different. Furthermore, the catalase activity under 20 µg/mL in control group in Alexandria, Rahaba, Agramia, Sahab showed an average (±SD) of 0.20±0.01, 0.39±0.01, 0.59±0.01, 0.53±0.04.

According to Two-way analysis of variance there were a highly significant difference in catalase activities induced by groups (p<0.001***), sites (p<0.001***), concentrations (p<0.001***). Moreover, the interaction between groups and sites showed a highly significant difference.

Table (4): The catalase activities (CAT, U/mL) estimated at different study sites (Alexandria, Rahaba, Agramia, and Sahab) at different concentration (10, 20 µg/mL), treatment groups (Control, Treated).

Conc. (µg/mL)	G	Catalase activity (Mean ± SD)			
		Alexandria	**Rahaba**	**Agramia**	**Sahab**
10	C	0.23±0.02	0.41±0.00	0.51±0.01	0.59±0.01
	T	0.33±0.03	0.42±0.02	0.55±0.01	0.59±0.01
20	C	0.20±0.01	0.39±0.01	0.59±0.01	0.53±0.04
	T	0.17±0.01	0.34±0.03	0.57±0.01	0.68±0.02
Univariate analysis					
Corr. model		**<0.001 *****	**Site x Group**		**<0.001 *****
Group		**<0.001 *****	**Site x conc.**		**<0.001 *****
Conc.		**<0.001 *****	**Group x conc.**		**<0.001 *****
Site		**<0.001 *****	**Site x Group x conc.**		**<0.001 *****

*, **, *** significant at p<0.05, <0.01, <0.001, NS, nonsignificant at p>0.05

5- Lipid peroxidation (MDA)

The oxidative stress in terms of lipid peroxidation (Umol/L mol d. MDA) recorded at different study sites (Alexandria, Rahaba, Agramia, and Sahab) at different concentration (10, 20 µg/mL), treatment groups (Control, Treated) were presented in table **(5).**

The MDA level was significantly (p<0.05) increased in treated group as compared to control group as revealed by ANOVA in Alexandria, Rahaba, Agramia, Sahab. The MDA level under 10 µg/mL in control group in Alexandria, Rahaba, Agramia, Sahab showed an average (±SD) of 3.34±0.45, 7.86±0.16, 10.04±0.21, and 1.20±0.45, however, treated groups showed a higher level recorded an average (±SD) 6.14±0.58, 13.96±1.24, 11.66±0.44, and 1.50±0.50. Furthermore, the MDA level under 20 µg/mL in control group in Alexandria, Rahaba, Agramia, Sahab showed an average (±SD) of 8.28±0.35, 16.86±0.74, 6.40±0.55, and 3.10±0.68 however, treated groups showed a higher level recorded an average(±SD) of 9.37±0.27, 18.78±0.61, 5.40±0.55, and 2.92±0.46; respectively. According to Two-way analysis of variance there were a highly significant difference in MDA level induced by groups (p<0.001***), sites (p<0.001***), concentrations (p<0.001***).

Table (5): The oxidative stress in terms of lipid peroxidation (Umol/L mol d. MDA) recorded at different study sites (Alexandria, Rahaba, Agramia, and Sahab) at different concentration (10, 20 µg/mL), treatment groups (Control, Treated).

Conc. (µg/mL)	G	Lipid peroxidation level (Mean ± SD)			
		Alexandria	Rahaba	Agramia	Sahab
10	C	3.3±0.45	7.9±0.16	10.0±0.21	1.2±0.45
	T	6.1±0.58	14.0±1.24	11.7±0.44	1.5±0.50
20	C	8.3±0.35	16.9±0.74	6.4±0.55	3.1±0.68
	T	9.4±0.27	18.8±0.61	5.4±0.55	2.9±0.46
Univariate analysis					
Corr. model		<0.001 ***	Site x Group		<0.001 ***
Group		<0.001 ***	Site x conc.		<0.001 ***
Conc.		<0.001 ***	Group x conc.		<0.001 ***
Site		<0.001 ***	Site x Group x conc.		<0.001 ***

, **, * significant at p<0.05, <0.01, <0.001, NS, nonsignificant at p>0.05.*

6- The Protein Carbonyl

The Protein Carbonyl (nmol/mg-protein) level recorded at different study sites (Alexandria, Rahaba, Agramia, and Sahab) at different concentration (10, 20 µg/mL), treatment groups (Control, Treated) were presented in table **(6).**

The Protein Carbonyl level was significantly (p<0.05) increased in treated group as compared to control group as revealed by ANOVA in Alexandria, Rahaba, Agramia, Sahab. The Protein Carbonyl level under 10 µg/mL in control group in Alexandria, Rahaba, Agramia, Sahab showed an average (±SD) of 0.26±0.008, 0.31±0.014, 0.53±0.008, and 0.54±0.032, however, treated groups showed a higher level recorded an average (±SD) 0.21±0.009, 0.22±0.014, 0.60±0.011, and 0.44±0.015. Furthermore, the Protein Carbonyl level under 20 µg/mL in control group in Alexandria, Rahaba, Agramia, Sahab showed an average (±SD) of 0.20±0.010, 0.19±0.004, 0.52±0.018, and 0.55±0.024, however, treated groups showed a higher level recorded an average(±SD) of 0.21±0.013, 0.23±0.014, 0.62±0.013, and 0.30±0.014; respectively. According to Two-way analysis of variance there were a highly significant difference in protein Carbonyl level induced by groups (p<0.001***), sites (p<0.001***), concentrations (p<0.001***).

Table (6): The Protein Carbonyl (nmol/mg-protein) level recorded at different study sites (Alexandria, Rahaba, Agramia, and Sahab) at different concentration (10, 20 µg/mL), treatment groups (Control, Treated).

Conc. (µg/mL)	G	Protein carbonyl (Mean ± SD)			
		Alexandria	Rahaba	Agramia	Sahab
10	C	0.3±0.008	0.3±0.014	0.5±0.008	0.5±0.032
	T	0.2±0.009	0.2±0.014	0.6±0.011	0.4±0.015
20	C	0.2±0.010	0.2±0.004	0.5±0.018	0.6±0.024
	T	0.2±0.013	0.2±0.014	0.6±0.013	0.3±0.014
Univariate analysis					
Corr. model		<0.001 ***	Site x Group		<0.001 ***
Group		<0.001 ***	Site x conc.		<0.001 ***
Conc.		<0.001 ***	Group x conc.		<0.001 ***
Site		<0.001 ***	Site x Group x conc.		<0.001 ***

, **, * significant at p<0.05, <0.01, <0.001, NS, nonsignificant at p>0.05.*

7- NO

NO level recorded at different study sites (Alexandria, Rahaba, Agramia, and Sahab) at different concentration (10, 20 µg/mL), treatment groups (Control, Treated) were presented in table **(7)**.

The NO level was significantly (p<0.05) increased in treated group as compared to control group as revealed by ANOVA in Alexandria, Rahaba, Agramia, Sahab. The NO level under 10 µg/mL in control group in Alexandria, Rahaba, Agramia, Sahab showed an average of 11.20±0.80, 91.14±0.96, 69.70±1.68, and 71.78±0.80, however, treated groups showed a higher level recorded an average 14.70±1.28, 73.10±8.25, 70.06±1.31, and 73.16±3.19. Furthermore, the NO level under 20 µg/mL in control group in Alexandria, Rahaba, Agramia, Sahab showed an average of 10.12±1.37, 29.84±0.40, 70.40±1.14, and 72.42±1.58, however, treated groups showed a higher level recorded an average(±SD) of 30.00±3.21, 64.84±0.67, 70.60±1.14, and 70.56±1.16; respectively.

Table (7): NO level recorded at different study sites (Alexandria, Rahaba, Agramia, and Sahab) at different concentration (10, 20 µg/mL), treatment groups (Control, Treated).

Conc. (µg/mL)	G	NO (Mean ± SD)			
		Alexandria	Rahaba	Agramia	Sahab
10	C	11.2±0.80	91.1±0.96	69.7±1.68	71.8±0.80
	T	14.7±1.28	73.1±8.25	70.1±1.31	73.2±3.19
20	C	10.1±1.37	29.8±0.40	70.4±1.14	72.4±1.58
	T	30.0±3.21	64.8±0.67	70.6±1.14	70.6±1.16
Univariate analysis					
Corr.model		<0.001 ***	Site x Group		<0.001 ***
Group		<0.001 ***	Site x conc.		<0.001 ***
Conc.		<0.001 ***	Group x conc.		<0.001 ***
Site		<0.001 ***	Site x Group x conc.		<0.001 ***

, **, * significant at p<0.05, <0.01, <0.001, NS, nonsignificant at p>0.05.*

Interaction between variables:

The effect of each independent variable (factor) and interaction between variables were presented in table 9 which summarizes the Multivariate analysis of variance table (MANOVA). According to MANOVA, Almost all corrected models were highly significant for hemoglobin, glutathione, SOD, CAT, Lipid peroxidation, carbonyl proteins, and NO. Moreover, there were a highly significant difference as induced by groups (p<0.001***), sites (p<0.001***), concentrations (p<0.001***).

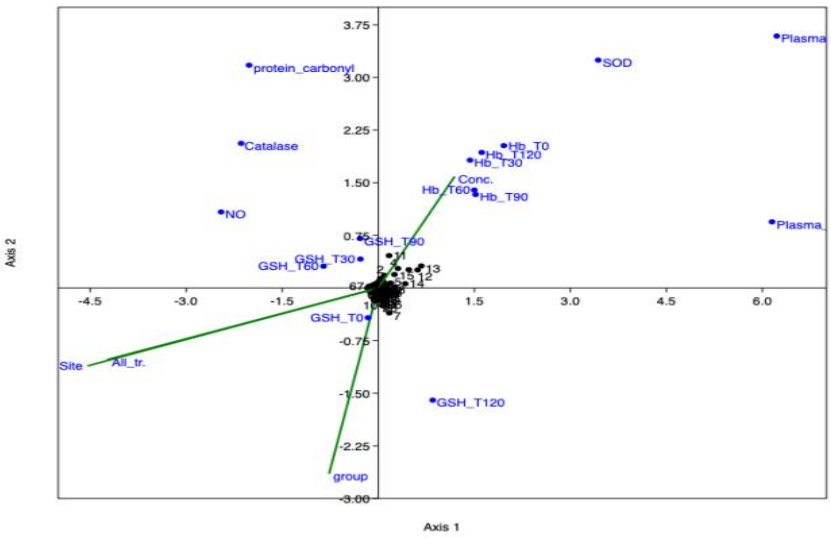

Figure (2): *PCA ordination showing the interaction between study variables.*

DISCUSSION

We have studied the cytotoxicity induced by the scorpion venom of Scorpio mauruspalmatus inhabiting different localities in Egypt, and found that our findings showed cytotoxicity.

The paramaters of this study could be classified into oxidative stress of lipid peroxidations malonaldehyde MDA biomarkers and protein carbonyls PC. On the other hand,antioxidants as Glutathione GSH, nitric Oxide NO, super oxide dismutase SOD, and Catalase CAT. In addition to the hemoglobin level.

Our findings showed that hemoglobin Hblevel showed that the level of Hb is close or similar to control group at concentration of 10ug/ml, except in Sahab site. Also, at 20ug/ml concentration the Hb level was increased at different time points. The results from this study showed that the glutathione level in Sahab group increased at 20 ug/ml concentration. The SOD activity was significantly increased as compared to control group.

We found that The SOD concentration at 20 ug/ml increased in the entire groups of the study, and Alexandria is the most higher one.The Catalase activity was significantly increased in treated groups at 20 ug/ml in Sahab group.The MDA marker is increased at the concentration of 10ug/ml in all sits but Rahaba is the most remarkable one. Moreover, is higher at 20 concentrations as compared to control. The PC increased at the concentration of 20ug/ml in only in Alagramia site, as shown in its table. Our results indicated that the concentration of NO at 10ug/ml is increased in all sites except Rahaba location. However, Rahaba was increased to the double of the control group. Also, Alexandria was remarkably increased to the triple of thecontrol group as well.

The results presented here showed that the crude venom of scorpion mauruspalmatus collected from different geographical regions in Egypt induced an obvious in vitro effect.There are two polypeptide toxins (maurotoxin and maurocalcine) are thought to be behind the toxicity of the venom.

Levels of glutathione, lipid peroxidation, protein carbonyl content and nitric oxide, as well as activities of superoxide dismutase, catalase were measured to assess the cytotoxicity of the venom.

Maurotoxinis a short peptide toxin containing 34 amino acid residues isolated from the scorpion Scorpiomauruspalmatusis behind the cytotoxicity effect ***(Kharrat et al., 1997)***. Our study showed that increase in MDA especially in 20 ug.ml concentration. On the other hand, Previous studies indicated that the concentration of lipid peroxidation isreduced and might be affected by metabolic rate of the organisms**.**

Our findings are partly consistent with previous studies ***(Abdel-Rahman et al., 1999).*** in regards to increase in nitric oxide (NO) concentration in the treated groups of Rahba and Alexandria, and their decrease in lipid perioxidation level could be attributed to the significant increase in NO, because it acts

as stress marker.Previous studies have confirmed that Nitric oxide (as a free radical scavenger) has been shown to inhibit lipid peroxidation in cell membrane ***(Rubboet al., 2002)***.

No (as a free radical scavenger) has been shown to inhibit lipid peroxidation in cell membranes ***(Rubbo et al., 2000).*** On the other hand, we observed anincrease in the level of nitric oxide.Our results are consistent with previous studies which reported that increased carbonyls have been confirmed and reported in several chronicdiseases, as in both ischemic heart diseases and skeletal muscle insult due to vigorous exercise ***(Butterfield and Kanski, 2001)***. So the increase in protein carbonyls might be in part due to the increase in nitric oxide (NO) concentration.

Nitric oxide is the major endothelium-derived relaxing factor (EDRF**).** In the current study, we suggested that the NO may play a prominent protective role against the toxic effects of MTX and MCa toxins. The presence of two polypeptide toxins (maurocalcine and maurotoxin) in this specific venom. Maurocalcine (MCa) is a 33 amino acid residue peptide that was isolated from the venom of the scorpion S. m. palmatus ***(Boisseau et al., 2006).***

In agreement with Previous studies have shown that increased carbonyls have been detected in several cases such as skeletal muscle damage due to exhaustive exercise ***(Touyz, 2004)***. In agreement with the study of **Reys *et al., (2002)***, we detected an increase in the level of NO in Rahaba and Alexnadria Groups.

This study is an attempt to elucidate the mechanisms of cytotoxicity induced by the venom of scorpion mauruspalmatus, and we found that the increased Hb level with different time points, especially in 20ug/ml, due to toxic effect of the venom on the RBCs. Therefore, RBCs have become fragile and Hb level increased.

In agreement with ***(Hafiz et al., 2015)***, the venom of Odontobuthusodonturus scorpion caused drastic change in blood physiology of treated animals. We observed increased in hemoglobin levelin the study.This kind of variation had been proved in the Egyptian scorpion species, Leiurusquinquestriatus and Scorpio mauruspalmatus collected from different geographical regions ***(Abdel Rahman et al., 2009).*** It is not clear whether intraspecific venom variation is a response to changes in diet ***(Mohamed and Omran 2012)***. Previous studies indicated that the concentration of lipid peroxidation (MDA) is affected by metabolic rate of the organisms ***(Zahida and Fatima, 2015)***. Moreover, the decrease in lipid perioxidation level could be attributed to the significant increase of nitric oxide (NO) concentration in the treated group of El- Agramia.

Intraspecific venom variation in S. m. palmatus may be due to geographic variation in diet. S. m. palmatus is distributed over an area (arid and semiarid) that encompasses diverse climatic regimens. Such climatic differences could be expected to result in differential distribution ofprey species and their relative abundance. If populations of scorpions start to diverge by virtue of geographical separation, intraspecific competition could contribute to divergence in toxin structure and would operate in parallel to other factors determined by interactions with the local prey ***(Abdel-Rahman, 2008)***.

Two types of diversity intraspecific and interspecific morphological and genetic characters of this species. The environmental conditions standing behind the structure and biological diversificationof this speciesor more probably an impression of the genetic variety and multiformity between and among different populace ***(Abdel-Rahman et al., 2009)***.

The scorpion of the study has been exposed to certain pressure, as in different scorpion population, to yield a variety of toxins. If scorpions population begin to diverge based on the geographic parting ***(Abdel-Rahman et al., 2009)***, intersex could add and enrich to molecular and genetic variety of toxins.

We suspect thata combination of local environmental conditions; geographical separation may play a major role in the intra-specific variation of venom of S.m. palmatus. Local ecological conditions and geographical parting could play a major role in the intraspecific variation of venom of S. m. palmate ***(Abdel Rahman et al., 2009)***. The temperature effect on metabolic rate has been

reported for at least 11 scorpion population in the literature confirming, a positive interrelation at the interspecific level for this group. More importantly, the variety between and among different species in the obliqueness of this relation gives support against the interspecific variation in relationship between scorpion Metabolic rate and temperature ***(Terblanche et al ., 2007).***

(Alqathani and Badry, 2020) considered that the separation may be connected tobiogeographical event during the segregation of the Arabian plate from the African plate. Collectively, it is suggested that increments in nitric oxide play, together with the direct action of venom toxins in various tissues. Intraspecific variability in the cytotoxic efficiency of the scorpion venom on the populations was observed. Principal component Analysis showed that scorpion venom of the Egyptian S. m. palmatus collected from different locations exhibits an intraspecific diversity in cytotoxic effects. Interestingly, the present toxicological findings are consistent with the molecular data on the level ofgenes and expressed protein of this scorpion species ***(Abdel-Rahman et al., 2009).***

Our findings consistent with a pervious study ***(Khazim, 2018)***, the venoms exerted cytotoxic effects on breast cell lines in a dose- and time-dependent manner. Enhanced apoptotic cells, increase in reactive oxygen species, and cell cycle arrest were observed after challenging these cell lines with scorpion venoms.

It is worth mentioning that some scorpion venom using and application in the field of therapy and medications.Venom of scorpions is very costly and fatal, yet with countless and potential source for designing of therapeutics. One of the main biological structures of scorpion venom is the molecular 'peptides' which have shown diverse of biological activities, and regulation **(*Suhas, 2022*).**

It has been reported and documented that some pathobiochemical conditions as generation of free radicals may initiate the beginning of organsfailure. One of the mechanisms which venom induces oxidative stress through reduction of antioxidant systems and alters some pathophysiological factors of envenomed mice ***(Milad et al., 2017).***

NO readily reacts with superoxide (O2) to form peroxynitrite (ONOO), a potent oxidant and nitrating agent capable of attacking and modifiying proteins, as well as depleting antioxidant defences ***(Joe, 2000).*** The cytotoxic effects of scorpion venom could be attributed to the generation of reactive oxygen species causing oxidative damage ***(Abdel Rahman et al., 2018)***.

Collectively, S. m. palmatus venom has the ability to induce oxidative stress in treated groups manifested by increase in the level of NO and Protein content concentration.

Limitations of the study

The findings of this study have to be seen in the light of some limitations. Such as the following points.

1- Chronotoxicology

Study of the influence of biological rhythms on the toxicity of substance. Biorhythmicity of living organisms is well for many decades in numerous studies in cell biology, in physiology and more recently in pharmacology and toxicology. Reality of temporal changes in structures and functions of biological systems is clear whatever their complexity level. Such temporal variations can explain that the same toxic does not induce the same efficacy in living organisms, if it is given at different hours in the day or at different seasons in the year. This considers the classical of the temporal dimension of the toxicology, so called chronotoxicity, describing the influence of time administration of drugs (toxic venom), may induce some variation on the experiments.

2- Milking or venom extraction.

a. Electrical method of extraction is an efficient method to get good quality and higher quantity of venom as compared to manual venom. However, we used the manual method of milking because we are not familiar with electrical method of extraction.

b. Manual extraction of venom

The telsonwere later separated from each scorpion and the venom was squeezed out into an Eppendorf tube using fine forceps. A watery secretion (prevenom) was collected and obtained followed via milky droplets (venom). One type produces the toxins, while

the other method produces just mucus. And that is why we have to milk the scorpion personally and not let the students milking the scorpion alone.

3- Collection of scorpions and their maintenance.

The collected scorpions might be from subspeciesthat is why we have to check after the hunter, to be certain that this is the species of the study. Effect of milking method, scorpion nutrition and temperature might have diversity on the venom production.However, the mentioned limitations did not affect the reliability of our findings

CONCLUSION

The venom of scorpion mauruspalmatus has significant cytotoxic effect via increasing the biomarkers stress, especially at 20ug/ml on different locations of the study.The findings presented here unfold the field for further research, particularly for the unrecognized components of the venom of Scorpio mautuspalmatus inhabiting different localities in Egypt.

RECOMMENDATIONS

1-Other experimental studies on the cytotoxicity of scorpiomauruspalmatus with different methods.

2- Further studies should be conducted on other antioxidants and investigate its effect with different assays.

3- Further studies to link cytotoxic effect of the scorpion venom as novel therapeutic agents against malignant and chronic disorders.

4- The findings of our study unfold the field of drug design for further research and investigation.

5- Scientific and medical research in both basic and clinical sciences often related to medical application. Our cytotoxicity findings could be used as selective toxicity in certain harmful cells such as refractory and chronic disorders. This is a promising research area in the field of drug design, as the active molecules as well as with their mechanism of action could beinvolved in developing therapeutic agents, particularly anticancer drugs. Malignancy is the expected area of application, because cancer is still the main threat to humans.

6- The use of scorpion venom in the process of drug design as a therapeutic agent against refractory disorders is an important point, not only for its immediate impact but also for the long termapplication and implications. Hopefully, and medical doctorsand experts in world Health Organization will now plan concerned joint action for both vaccination design andbiotherapeutics aiming at curing the chronic and malignant disorders.

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السمية الخلوية المستحثة بواسطة سم العقرب (Scorpio mauruspalmatus) المنتشر فى مناطق مختلفة بمصر

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الملخص العربى

المقدمة: عقرب palmatusmaurusscorpio ينتمى الى عائلة الscor-pionidae وشائع فى السعودية ,ومناطق الشرق الاوسط, وساحل البحر المتوسط ومناطق الاردن. موجود اصلا فى مصر, فى وادى النطرون والقاهرة والفيوم, وجنوب ووسط سيناء.الهدف من الدراسة: دراسة السمية الخلوية المستحثة بواسطة سم العقرب موضوع الدراسة.
طريقة الدراسة: تجميع العقارب واعداد عينات السم. الحصول على العقارب من أربع مناطق, الاسكندرسة, رحابة, سحاب, والعجرمية. تم حفظ العينات بشكل منفصل, كل مجموعة فى حاوية خاصة بها.تم حلب العقارب باستخدام اسلوب الضغط على العقرب. وعينات سم العقارب تم تجميعها وتخزينها فى 20 درجة تحت الصفر قبل الاستخدام.التجارب الخاصة بالبارمتر باكتشافالجذور الحرة الناتجة عن الاكسدة .أيضا المالون دايالدهايد وايضا القياسات الخاصة بحماية الخلية ضد الاكسدة مثل النيتريك أوكسيد, والكاتاليز , الجلوتاثيون والنحاس والزنك SOD. هذا بالاضافة الى تقدير نسبة الهموجلوبين.
تصميم الدراسة: لدراسة سم عقربscorpiomurauspalmatus, تم تجميع السم من مناطق مختلفة فى مصر, أيضا تم تجميع عينات الدم من عشرون فئر مخدر, بواسطة عينة من التجويف العينى. ,تم تقسيم عينات الدم الى تركيزين من سم العقرب تحت الدراسة 10, 20 ميكروجرام لكل مل.
النتائج: أظهرت زيادة نسبة الهيموجلوبين بشكل معنوى $P<0.05$ وذلك باستخدام اختبار تحليل المتغيرات. بالنسبة للجلوتاثيون هناك فرق معنوى بالنسبة للمجموعات والتركيز والاماكن. ال $P<0.05$ يزيد بشكل معنوى مع الوقت وذلك من خلال تحليل التباين فى مناطق الاسكندرية,ورحبة, والعجرمية, ومع ذلك تقل نسبته فى مجموعة سحبة. و هناك فرق معنوى بالنسبة لنشاط ال SOD بالنسبة للمجموعات والاماكن والتركيز. وأيضا النيتريك أوكسيد هناك زيادة معنوية حيث أن $P<0.0.05$ فى المجموعات, خاصة فى منطقتى الاسكندرية ورحاب, عند تركيز 20ميكروجرام. أيضا كلا من ال MDA-PC هناك زيادة ملحوظة فى كلا التركيزين, فى كل من الاسكندرية ورحابه والعجرمية. وبالنسبة للكلتاليز يزيد فى منطقة سحاب عند 20 ميكروجرام.
الخلاصة: من هنا يتضح أن سم عقرب الpalmatusmauruscorpio له سمية خلوية وذلك باكتشاف الجذور الحرة ,وايضا عند زيادة النيترك أوكسيد , ويمكن اعتباره احد الكواشف الحيوية للاكسدة