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EFFECT OF THE EGYPTIAN SAHARA SAND VIPER VENOM ON HUMAN BREAST CANCER: AN *IN VITRO* STUDY

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Abstract

Cancer is a major health problem which is considered as one of the most reasons of death worldwide. Breast cancer is a second cause of women death and estimated to have 2.1 million new cases. Traditional treatment of cancer based on different clinical approaches is included surgery, chemotherapy, radio therapy, hormone therapy and combination therapy which affect both cancer and normal cells. The present study investigated the anticancer potential of *Cerastes vipera* venom in comparison with Cisplatin drug on MDA-MB-231 breast cancer cell line. Cytotoxicity activity of tested material was evaluated using MTT assay. Cell cycle arrest was performed using propidium iodine DNA staining. Apoptotic detection was determined using annexin V-FTIC and for the detection of the genes Caspase 3, Caspase 8 & Caspase 9 were determined by ELISA. The *C. vipera* venom, Cisplatin and PLA2 had IC50 as 3.4, 10.3, 35.1 µg/mL with MDA-MB-231 cell line respectively. Cell cycle has arrest of cells in G2/M of *C. vipera* venom and PLA2. Tested venoms have apoptotic effect against MDA-MB-231 cell line through elevation of Caspase 3, Caspase 8 & Caspase 9 genes.

Keywords: Breast cancer, Snake venom, MDA-MB-231, *Cerastes vipera* venom, Phospholipase A2 (PLA2), and Cisplatin.

Introduction

Breast cancer in women is a major health burden. It is the most common cause of cancer death among women impacting 2.1 million female every year (WHO, 2018). In Egypt, breast cancer standardized in a second rank. The incidence rate of breast cancer was calculated and estimated that approximately 38.7% were among women and 21.6% among both sexes. National Cancer Registry Program (NCRP) estimated that a threefold increase of incidence rate of in 2050 relative to 2013 (Ibrahim *et al*, 2014). Nowadays, treatment of Breast cancer is a fundamental challenge to the medical world. Present strategies of treatment are very expensive and have several side effects. Patient has to suffer physically and mentally. Some of the elements of snake venom reason retardation of growth of cancerous cells. Due to its therapeutic activity, potency and availability, snake venom may also be a fundamental nominee for the remedy in the future for many diseases and disorders. Viewing and analyzing with futuristic prospectus in the pharmaceutical world, snake venom could open the doorways for new generation of the medicines and research for treatment of Breast cancer (Bazaa *et al*, 2005). *Cerastes vipera* venom contains a complex mixture of enzymes including phospholipaseA2 and proteases, L-amino acid oxidase, C-type lectin and Disintegrins. Especially PLA2 which is considered the main component of the vipera venom causes hydrolysis of the membrane phospholipids which induces the release of lysophospholipids and fatty acids resulting in the pharmacological effects within cells such as membrane damage, disruption of membrane-bound protein and functional disturbances of the cellular cascade, and liberation of some bioactive products (El-Aal *et al*, 2017). Snake venom inhibited cell proliferation and promotion of cell death by means of unique means: induction of apoptosis in cancer cell, increasing

Ca2+ influx; inducing cytochrome C release; decreasing or increasing the expression of proteins that control cell cycle; inflicting damage to cell membranes. Snake venoms include a significant array of components, the majority of which act on the peripheral nervous system for killing or immobilizing prey (Vyas *et al*, 2013).

The study aimed to improve a new agent from snake venoms as useful in cancer therapy especially breast cancer

Material and Methods

Cisplatin was diluted in RPMI-1641 media 1mg/1mL (BioWhittaker™ Classical Media, Lonza) to prepare test concentrations. *Cerastes vipera* venom or (Egyptian Sahara sand viper) venom (*C.v.*), phospholipase A2 (PLA2) were kindly supplied from ANDI Center, VACSERA. They were dissolved in 1mg/1mL sterile distilled water and sterilized using 0.22m disposable syringe filter (Millipore-USA) and serially diluted in the RPMI-1641 media (BioWhittaker™ Classical Media, Lonza). MDA 321 (human breast cancer cell line) was supplied from American Type Culture Collection (ATCC). Trypsin 0.25% % was provided by VACSERA-Cell Culture Unit, Egypt. Cells were seeded at a density of 2×10^5/ml and routinely cultured in RPMI-1641 medium in tissue culture flasks (Grien-er, Germany).

Cytotoxicity by using colorimetric assay (MTT): MDA 321cell line was seeded in 96-well tissue culture plate with 100µL of the RPMI medium. 200µL tested materials serial dilution using two fold dilutions. Plates were incubated with the venom concentrations of for 24h at 37ºC. Plates were read using ELISA reader at wave length 570nm and the absorbance was correlated with cell number. Cytotoxic effects of the tested venom on the MDA 321 cell lines were expressed as IC_{50} value. Inhibitory concentration (IC_{50}) was assessed by the Masterplex software (2010).

Flow cytometry cell cycle analysis using Propidium iodide DNA staining: For cell cycles analyzed by FACS, cell line was seeded in DMEM with 10% FBS, fixed in cold 70% ethanol on 6-well plates and then allowed to attach overnight. While, the vortexing, pellet was added drop wise. All cells were fixed for 30min at 4^oC and minimized clumping. After fixation, cells were washed in PBS and spun at 850g in a centrifuge. Supernatant was discarded especially after ethanol spinning out. The cells were treated with ribonuclease I (100mg/ml stock solution). 50µl of 100µg/ml sock of RNase was added to ensure that only the DNA and not RNA were stained. 200µl propidium iodide was added (50µg/ ml stock solution).

Apoptosis analysis by using Annex in V-FITC assay: $1\text{-}5x10^5$ cells were collected by centrifugation and were re-suspended in 500µl of 1X Binding Buffer. 5µl of Annexin V-FITC & 5µl of propidium iodide (PI 50 mg/ml) were added. Incubation was performed at room temperature for 5min in the dark. Annexin V-FITC binding was analyzed by flow cytometry (Ex= 488nm; Em = 530nm) using FITC signal detector and PI staining by phycoerythrin emission signal detector. For adherent cells, they were gently trypsinized and washed once with serum-containing media before incubation with Annexin V-FITC. Stained cells were analyzed for percentage of apoptotic cells by Partec flow cytometer and FloMax software. Casps 3, 8, & 9 were detected by ELISA using anti-human caspase coating antibody adsorbed onto micro-wells. Human caspase present in the sample or standard binds to antibodies adsorbed to the micro-wells. The polyclonal detection antibody (rabbit) binds to human caspase captured by the first antibody. Following incubation unbound detection antibody was removed during a wash step. Anti-rabbit-IgG-HRP was added and binds to the antibody detection. After incubation unbound anti-rabbit-IgG-HRP was removed during a wash step, and substrate solution reactive with HRP is added to the wells. A colored product is formed in proportion to the amount of human Caspase-9 present in the sample or standard. The reac-

tion is terminated by addition of acid and absorbance was measured at 450nm. The standard curve was prepared from human caspase standard dilutions and human caspase concentration determined.

Statistical analyses: Experiments were carried out in triplicate unless specified. Results were represented as mean ± SEM. Analysis was done using unpaired Student's t test. Multiple groups were compared by one-way ANOVA with Dennett's post-test. P value < 0.05 was considered significant & p < 0.01 was highly significant.

Results

The results were shown in tables (1, 2, 3, 4, 5) and figures (1, 2, 3, 4, 5 & 6)

Table 1: *Cerastes vipera* venom against Cisplatin on inhibitory effect on MDA-MB-231 breast cancer cell line.

Series	Sample code	IC50uM
		MDA-MB-231
1	Snake venom	3.480960187 ±0.17
2	PLA2	35.10247153 ±1.6
3	Cisplatin	10.36281351 ±0.52

Table 2: Effect of cell cycle concentration on MDA-MB-231 cell line by different samples

Sample data		Results				
Series	Sample code	%G0-G1	%S	%G2-M	%Pre-G1	Comment
1	Snake venom	23.76	19.73	56.51	27.52	PreG1apoptosis&Cell growth arrest@G2/M
2	PLA2	43.88	25.06	31.06	13.25	PreG1apoptosis&Cell growth arrest@G2/M
3	Cisplatin	27.82	17.59	54.59	24.71	PreG1apoptosis&Cell growth arrest@G2/M
4	Cont cancer cell	61.54	28.29	10.17	1.41	

Table 3: Evolution of viper venom, PLA2 and Cisplatin on MDA-MB-231 cell line.

Series	code	Apoptosis			Necrosis
		Total	Early	Late	
1	Viper venom	27.52	7.58	16.67	3.27
2	PLA2	13.25	4.39	6.25	2.61
3	Cisplatin	24.71	5.93	14.85	3.93
4	Cont	1.41	0.76	0.28	0.37

Table 5: Gene expression level of Casp3 in treated cells and untreated cells

Series	Cpd. code.	Caspase3	
		MDA-MB-231	
		Pg/ml	Fld
1	Viper venom	476.9 ±19.6	7.47
2	PLA2	225.1 ±11.02	3.52
3	Cisplatin	347.4 ±14.6	5.44
4	Cont	63.83 ±3.4	1

Table 6: Gene expression level of Casp8 in treated cells and untreated cells

Compound		Results		
			MDA-MB-231	
series	Cpd. code.	Assay conc. uM	Caspase8	
			ng/ml	fld
1	Snake venom		0.6861 ±0034	3.31
2	PLA2		0.5004 ±0.02	2.41
3	Cisplatin		0.612 ±0.03	2.95
4	Cont		0.2068 ±0.01	1

Table 7: Gene expression level of Casp (in treated cells and untreated cells

Sample		Results	
Series	Cpd.code	Caspase 9 ng/ml	
		MDA-MB-231	fld
1	Snake venom	30.18 ±1.42	18.89
2	PLA2	14 ±0.66	8.76
3	Cisplatin	23.38 ±1.13	14.63
6	Cont	1.597 ±0.05	1

Discussion

There was a significant advancement in cancer therapy investigation which involved synthesis of peptides and proteins specific for oncoproteins (Chaubey, 2014). Subsequently, after realizing the potential of medical use of peptides and proteins as anticancer effects, several proteins and peptides are isolated from animal origin. According to Chaisakul *et al.* (2016) venoms of several animal species including snake and scorpion as well as their biochemical derivatives have investigated in the potential therapeutic tool as anticancer effect. Regarding to the previous studies of the complexity and cytotoxicity of bioactive components of animal venoms, they have characterized through highly levels of specificity leads to induce various types of tumor cells by interfering with the mechanisms of hallmarks in cancer development (Gomes *et al,* 2010; Alyan *et al,* 2014; Abdel-Aziz *et al,* 2017). PLA2 are enzymes that catalyse the hydrolysis of sn-2 fatty acyl ester bonds of sn-3 phosphoglycerides, liberating free fatty acids and lysophospholipids. PLA2 are the most abundant protein found in vipers snake venoms, some venom PLA2s show antitumor by mechanism independent of their enzymatic activity (Zouari-Kessentini *et al,* 2013). Phospholipases A2 (PLA2) is enzymes of high medical scientific interest due to their involvement in a large number of human inflammatory diseases. PLA2 constitute a diverse family of enzymes which catalyzed the hydrolysis of the sn-2 ester bond in glycerophospholipids and exhibit a wide range of physiological and pathological effects. The ubiquitous nature of PLA2 highlights the important role they play in many biological processes, as cell signaling and cell growth, including the generation of proinflammatory lipid mediators such as prostaglandin and leukotrienes, regulation of lipid mediators. The activity and expression of several PLA2 isoforms are increased in several human cancers, suggesting that these enzymes have a central role in both tumor development and progression and can be targets for anti-cancer drugs. On the other hand, the some PLA2 isolated from Viperidae venoms are capable to induce anti-tumoral activity. In summary, PLA2 from snake venoms can be a new class of anticancer agents and provide new molecular and biological insights of cancer development (Rodrigues *et al,* 2009). However, because of drug resistance and numerous undesirable side effects such as severe kidney problems, allergic reaction, decrease immunity to infections, gastrointestinal disorders, hemorrhage, and hearing loss especially in younger patients, other platinum-containing anti-cancer drugs such as carboplatin, oxaliplatin and others, have also been used. Furthermore, combination therapies of cisplatin with other drugs have been highly considered to overcome drug-resistance and reduce toxicity, Its mode of action has been linked to its ability to cross-link with the purine bases on the DNA; interfering with DNA repair mechanisms, causing DNA damage, and thus inducing apoptosis in cancer cells (Shaloam and Paul Bernard, 2014). Salvesen and Dixit (1997) and Cummings *et al*. (2000) found that apoptosis is a controlled type of cell death that energy-dependent leading to cell shrinkage, chromatin condensation, membrane budding, hosphatidylserine externalization, and activation of a family of cysteine proteases called caspases. Caspase activation is the key step in the apoptosis beginning, and several stimuli activate caspases, including those that activate plasma membrane death receptors (caspase 8) and cause mitochondrial dysfunction (caspase 9). Caspases are either initiators or executioners of apoptosis. Initiator caspases include caspases 8 & 9, and activation of the caspases result in downstream activation or executioner caspases such as caspases 3 and 7 (Salvesen and Abrams, 2004). Executioner caspases are accountable in many of the biochemical characteristics of apoptosis, including cleavage and activation of poly (ADP-ribose) polymerase and of the inhibitor of caspase activator domain protein that leads

to DNA fragmentation.

In the present study, evaluation of cytotoxicity on *MDA-321* cells treated with *Cv* venom for 24hr, was 3.4µg/mL. This nearly agreed with Yalcin *et al*. (2014) and Bradshaw *et al*. (2016) who found that inhibitory concentration of Viperidae snake venoms toward *MCF-7* cells was after 24h incubation evaluated 4.2µg/mL. But, the result disagreed with Gomes *et al*. (2015) who found that cytotoxicity was 35.5µg/mL in case of russets viper on leukemic cancer cells after 48hr of treatment. This difference may be due to species difference and/or cell line used. Evaluation of *PLA2* cytotoxicity on *MDA 321* cells for 24hr was 35.1µg/mL. This result agreed with Attarde and Pandit (2017) who reported that Purified toxins from snake venoms was showed cytotoxic activity against *MCF-7* (ER+) and *MDA-MB-231* (ER-) cells via dose dependent manner. Also, Ravi *et al.* (2018) assured that the proapoptotic action of PLA2 isolated from snake venom towards leukemia cells, and explained that the cytotoxic mechanism of PLA2 are poorly under stood, this enzyme class directly act on the membrane phospholipid metabolism and interfere with lipid biosynthesis in tum- or cell line. Another possibility was that PLA2 triggers the production of reactive oxygen species and induces oxidative stress that associated with the cytotoxic effects (Marcussi *et al,* 2013). Depending on these results, that *Cv* venom was more toxic and potent than PlA2 fraction.

In the present study, cell cycle arrest of MDA321 treated *Cv* venom was observed in G2/M cell during incubation entering the subsequent stage and interrupted at this point. Thus, the anti-proliferative activity of the venom was detected which block cell growth in G2/M and induced apoptosis during Pre-G1. This result agreed with Gomes *et al.* (2010) and Chaisakul *et al.* (2017) who reported that the snake venom cytotoxins have an effect on targeting cancer cell proliferation, migration, invasion, neovascularization and apoptotic activity through arresting cell cycle in Pre-G1 population. But, this result disagreed with Gomes (2015) who showed that the Russell's viper venom arrested cell cycle in G1/S phase on leukemic U937 cancer cells after 24hr of treatment. This difference may be due to species difference and/or cell line used. Apoptosis of *Cv* venom was represented in early and late apoptosis as well as necrosis. This agreed with Ebrahim *et al.* (2014) who reported that MCF-7 cell lines treated with Cobra venom showed early apoptotic and late apoptotic as well as necrosis. This result may be explained by cytotoxicity of snake venom that targeted to cellular metabolism alterations and affected on cancerous cells by blocking of some specific ion channels, inhibiting angiogenesis and activating intracellular pathways causing apoptosis (Calderon *et al,* 2014; Ebrahim *et al,* 2016). Rahman and Choudhary (2015) reported that Viperistatin derived from Vipera venom has characterized a KTS motified in the integrin active binding site loop leading to block the α1β1 integrin. Thus, the antiangiogenic cyclic KTS peptides were induced the blockage of the binding of α1β1 and α2β1 integrins.

In the present study, cell cycle arrest of MDA321 treated PLA2 showed that G2/ M cell during incubation entering the subsequent stage and interrupted at this point. As a result, the anti-proliferative activity of the venom was detected which block cell growth in G2/M and then induced apoptosis during Pre-G1. Apoptosis of the PLA2 venom was represented in early and late apoptosis as well as necrosis, but was lower than *Cv* and stander drug. Chaisakul *et al.* (2016) found that other active components of snake venom such as PLA2 have the potential of anticancer effects. PLA2 was induced apoptosis in various cancer types such as breast adenocarcinoma, human leukemia T and Erlich ascetic tumor cell line. Omran (2003) reported that apoptosis included a cascade of cytoplasmic and nuclear events have been resulted in a series of morphological chang-

es and eventually cause cell demise through a variety of different stimuli. On the other hand, necrosis resulted in cell lysis and extensive damage to surrounding tissues by physical damage or oxygen starvation. Viability of the most sensitive cancer cell type was reduced upon exposure to the PLA2cells and to some extent also to PLA2s from the *V. lebetina* and *N. naja oxiana* (Khannoon *et al,* 2016). There was no inhibitory effect of all *Sv* PLA2 preparations towards LNCaP cells and low inhibitory effect (8% -20%) towards the PC-3, MCF-7 and B10-F16 cells (Samel *et al,* 2013).

In the present study, apoptosis and cell cycle arrest of MDA321 treated cisplatin showed G2/M cell during incubation entering the subsequent stage and interrupted at this point. The anti-proliferative activity of the venom was detected which block cell growth in G2/M and then induced apoptosis during Pre-G1. Cisplatin represents in early and late apoptosis as well as necrosis, these results nearly as *C.v.*, but gave more necrosis than our tested venom and fraction. This result agreed with Shaloam and Paul Bernard (2014) who explained that the mode of action was linked to its ability to crosslink with purine bases on the DNA; interfering with DNA repair mechanisms, causing DNA damage, and subsequently inducing apoptosis in cancer cells. There were two major pathways of the apoptotic cell death (Kischkel *et al,* 1995).

In the present study, Caspase 3, 8, & 9 showed gene expression level of effector gene as Caspase3 on MDA321 cell line after treatment with IC_{50} of *C.v.* venom by Real-Time PCR. Al-Sadoon *et al*. (2012) reported that Caspase-3 gene up regulation was detected in the treated MCF-7 with *Xanthina* venom. Thus, the two studies agreed as the results evaluate the up regulation of Capsase-3 gene in breast cancer cell lines treated with snake venoms leading to induce apoptosis. They added that induction of cell-cycle arrest in breast cancer cell lines was reported via caspase-dependent apoptosis by the snake venoms and their active compounds. The expression of active Caspase-3 was increased in MDA-MB-231 & MCF-7 breast cancer cells treated with snake venom. Animal venoms can alter the gene expression and increase the expression of pro-apoptotic proteins (Chaisakul *et al,* 2016). According to Calderon *et al*. (2014) snake toxins can increase the expression of pro-apoptotic proteins. PLA2 causing the lowest upregulation effect on caspase-3. This result agreed with Ravi *et al.* (2018) who explained that the loss of mitochondrial membrane potential and caspase 3 activations, and thus PLA2 induce apoptosis; also activation of caspase 3, & 9 confirmed the pro-apoptotic action of this toxin. Concerning Caspase 8, & caspase 9, showed that the gene expression level of theses initiator genes on MDA321 cell line after the treatment with the IC50 of venom using Real-Time PCR. Snake toxins can induce apoptosis via ROS-independent mitochondrial dysfunction pathway and the caspase-dependant mechanism (Calderon *et al,* 2014; Ebrahim *et al,* 2016). Moreover, the snake cytotoxins can contribute apoptosis mediating via cleaving poly (ADP-ribose) polymerase (PARP) (Gomes *et al,* 2010). Also, LAAOs of *Cv* stimulate apoptotic activity through the oxidative reaction mediated by releasing H_2O_2 in the oxidation process of the enzyme and causing alteration of gene expression that leading to trigger apoptosis (Gomes *et al,* 2010). Chaisakul *et al.* (2016) isolated LAAO from venom is exhibited a specific anticancer activity on human breast adenocarcinoma cell line by both extrinsic and intrinsic pathways leading to enhance the activities of caspase-8 & caspase-9 and then induce cell apoptosis. MDA-MB-231 cells treated with the PLA2 inhibitor from Viper venom show the lowest effect on caspase 8 & caspase 9, these results may be due to loss of mitochondrial membrane potential and caspase 3 activations. Thus, PLA2 induced apoptosis, and also activation of caspase 3, & 9 confirmed the pro-apoptotic action of this toxin (Ravi *et al,* 2018). MDA-

MB-231 cells treated with cisplatin showed lower effect than *Cv* on caspase 3, 8 & 9. This agreed with Shalaom and Paul (2014) who reported that the presence of two major pathways of apoptotic cell death. The extrinsic pathway was initiated when ligands bind to the tumor necrosis factor-α (TNFα) receptor super family followed by oligomerization and recruitment of procaspase-8 via adaptor molecules to form the death-inducing the signaling complex (Kischkel *et al*, 1995). The intrinsic pathway was initiated by cellular stress, such as DNA damage, resulting in release of cytochrome-c from the mitochondria causing activation of procaspase-9 via the interaction with apoptosis promoting activating factor-1 (APAF-1) and formation of an active apoptosome complex. Bcl-2 family proteins regulate DNA damage-induced apoptosis by regulating the release of mitochondrial cytochrome c in response to DNA damage. Cisplatin-induced genotoxic stress activa-ted the multiple signal transduction pathways, which can contribute to apoptosis or chemo resistance.

Conclusion

The outcome data proved that *Cerastes vipera* venom possesses anti-cancer potential on human breast cancer in comparison with Cisplatin drug. This positively related to the cell cycle arrest and apoptotic induction as well as apoptotic gene expression.

Recommendations

1- More cancer cell lines must be tested for verification of anti-cancer potentials of target test materials. 2- In vivo application of anti-cancer potentials of tested materials using murine models. 3- Monitoring of biochemical changes pre and post treatment with anti-cancer agent regarding the anti\oxidant parameters (GTH, MDA, NO, ROS etc.).

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Explanation of figures

Fig. 1: Determination of cytotoxicity (IC50) of *Cerastes vipera* venom, PLA2, and Cisplatin.

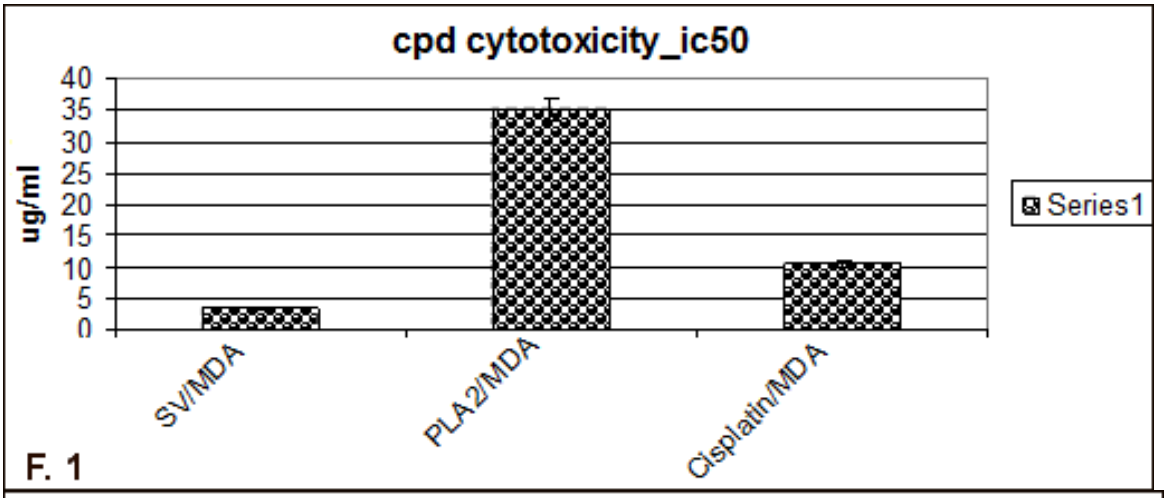
Fig. 2: Determination of cell cycle analysis under the effect of *C. vipera* venom, PLA2, and Cisplatin compared with control MDA-MB-231 cells.

Fig. 3: Determination of total, early &late apoptosis as well as necrosis under effect of *C. vipera* venom, PLA2m and Cisplatin com-pared with control MDA-MB-231 cells.

Fig. 4: Gene expression level of casp 3 under effect of *C. vipera* venom, PLA2 &Cisplatin compared with control MDA-MB-231 cells.

Fig. 5: Gene expression level of casp 8 under effect of *C. vipera* venom, PLA2 &Cisplatin compared with control MDA-MB-231 cells.

Fig. 6: Gene expression level of casp 9 under effect of *C. vipera* venom, PLA2 &Cisplatin compared with control MDA-MB-231 cells.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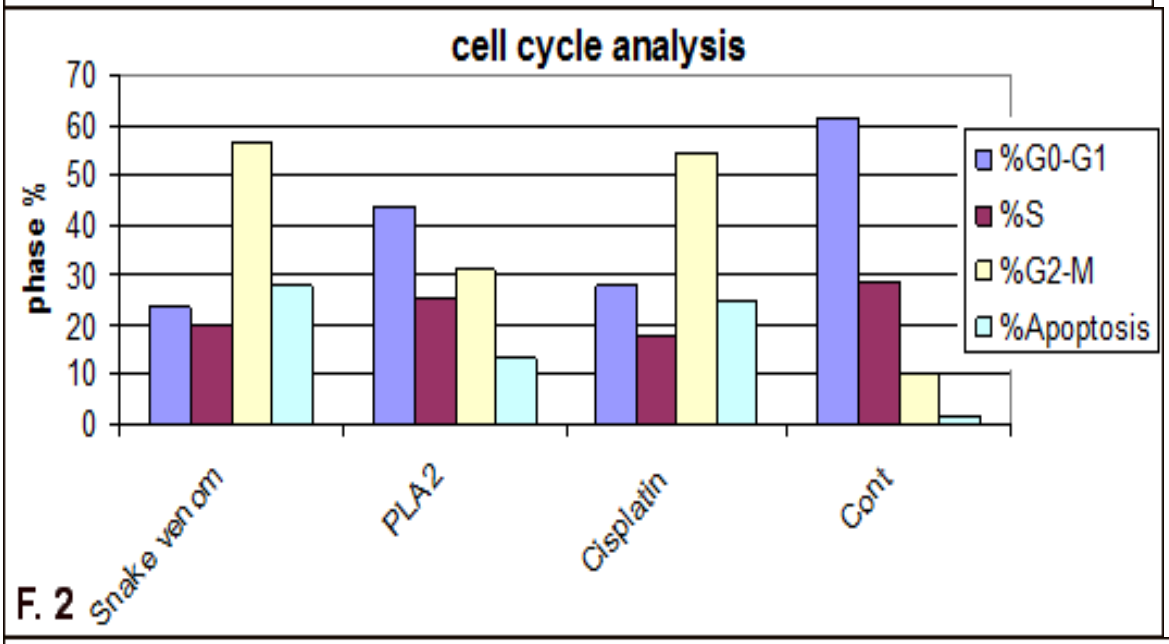
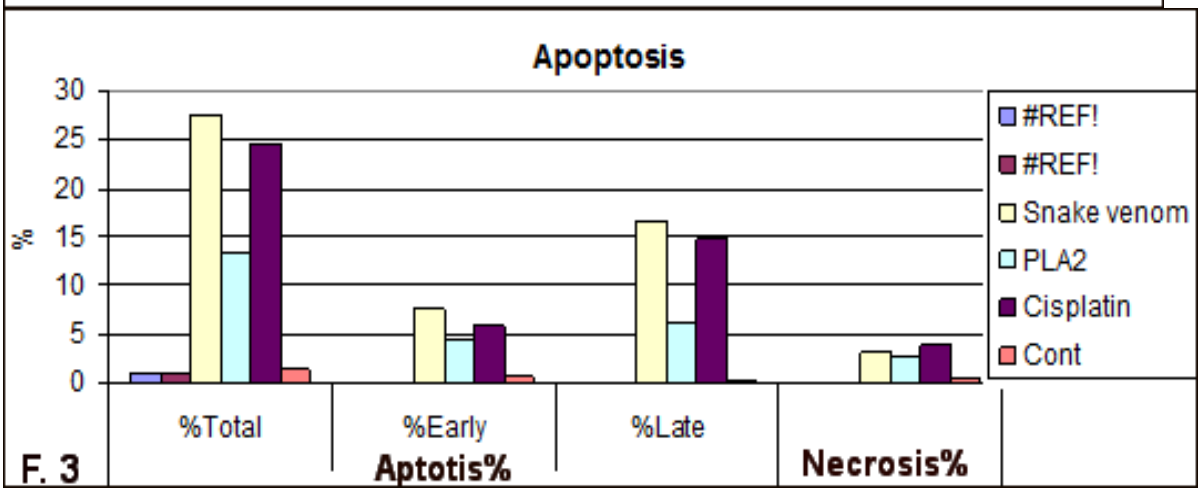
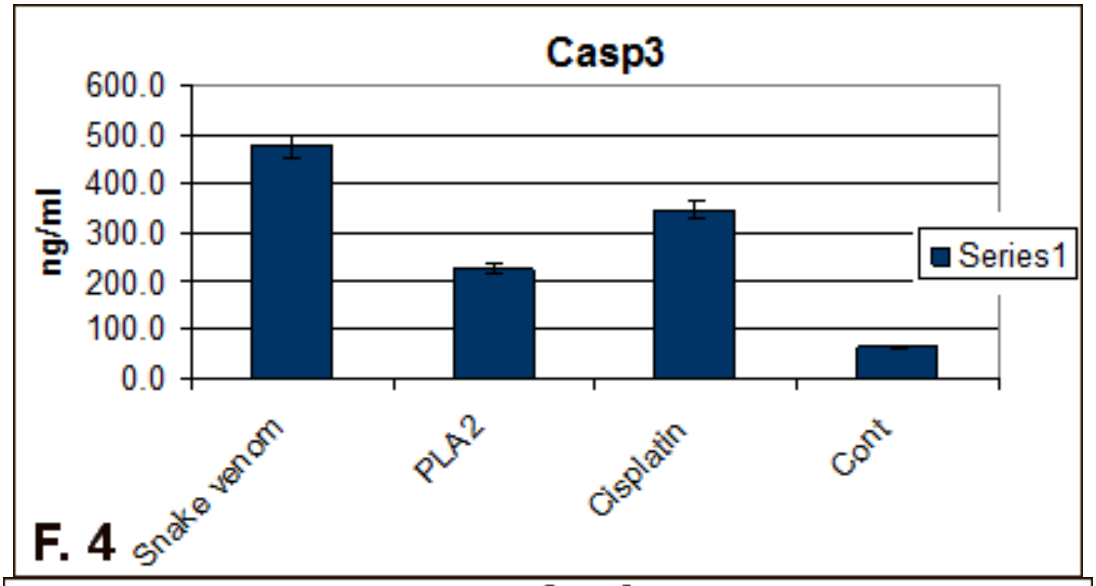

cell cycle analysis
phase %
70
60
50
40
30
20
10
0
Snake venom
PLA2
Cisplatin
Cont
%G0-G1
%S
%G2-M
%Apoptosis

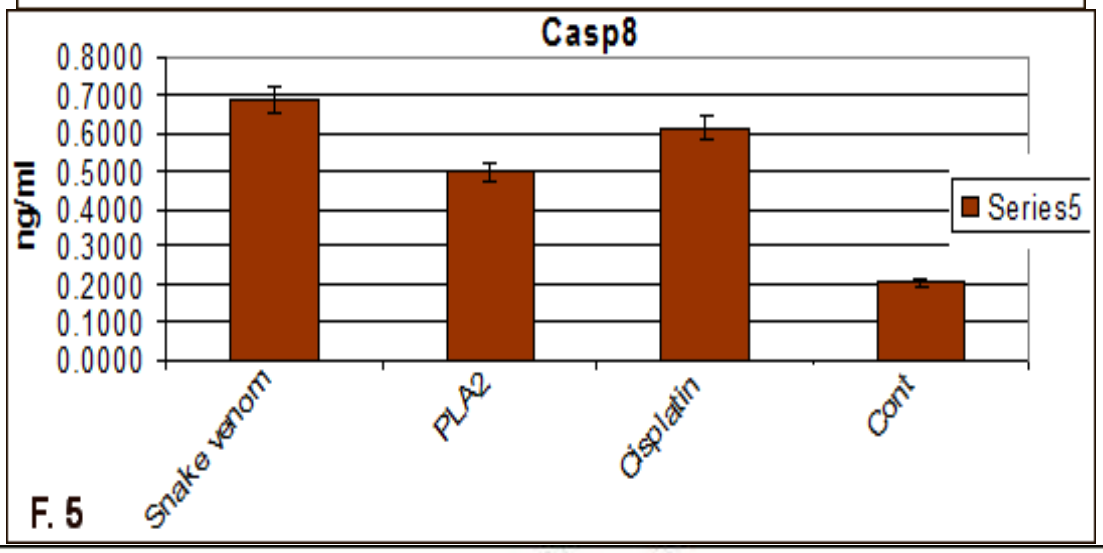
F. 2


Apoptosis
%
30
25
20
15
10
5
0
%Total
%Early
%Late
Aptotis%
Necrosis%
#REF!
#REF!
Snake venom
PLA2
Cisplatin
Cont

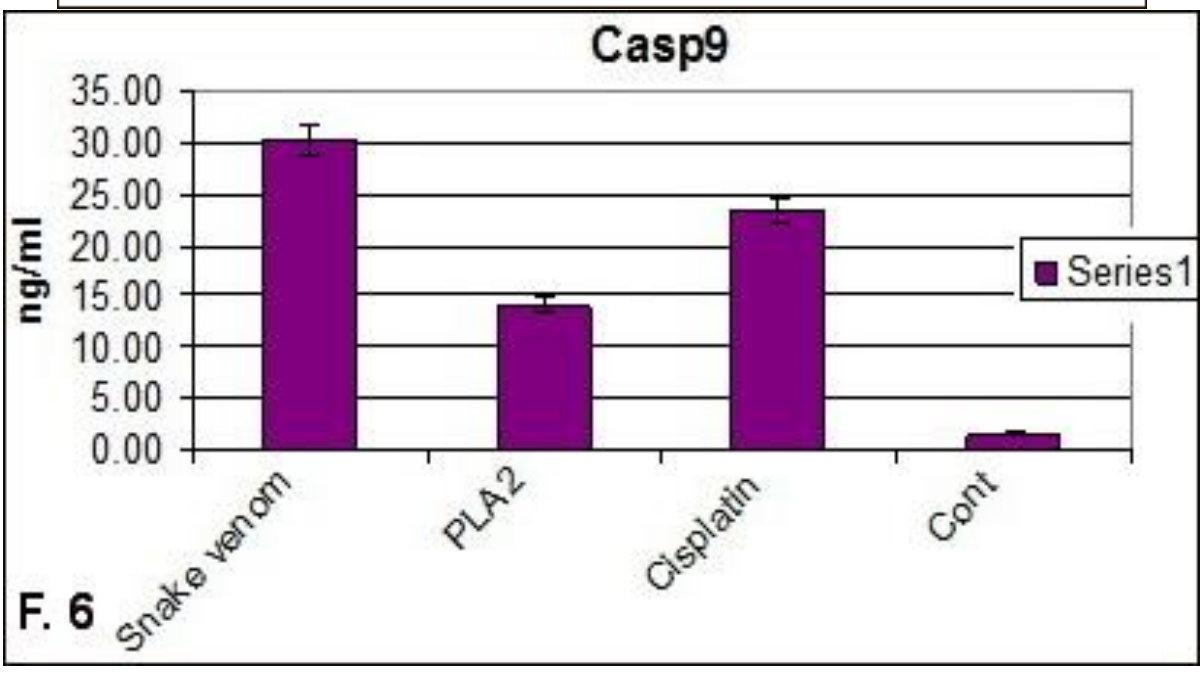
F. 3


Casp3
600.0
500.0
400.0
300.0
200.0
100.0
0.0
ng/ml
Series1
Snake venom
PLA2
Cisplatin
Cont

F. 4


Casp8
0.8000
0.7000
0.6000
0.5000
0.4000
0.3000
0.2000
0.1000
0.0000
ng/ml
Series5
Snake venom
PLA2
Cisplatin
Cont

F. 5


Casp9
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
ng/ml
Series1
Snake venom
PLA2
Cisplatin
Cont

F. 6