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INVESTIGATING THE EFFECT OF DIFFERENT ANIMAL VENOMS ON BREAST CANCER CELL LINES (*MCF-7*): *IN VITRO* STUDY

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Abstract

Traditional treatments of cancer include surgery, chemotherapy, radiotherapy, hormone therapy, and combination therapy which affect both cancer and normal cells. The present study investigated anti-cancer venom potentials of *Montivipera xanthina* and *Leiurus quinquestriatus* compared to the synthetic anti-estrogen drug (Tamoxifen®) on MCF-7 breast cancer cell line. Cytotoxicity of venoms was evaluated using MTT assay. Cell cycle arrest was done using propidium iodide DNA stained by Flow Cytometry. Apoptosis was determined using annexin V-FITC by Flow Cytometry. Gene expression analysis was done for Caspase3, HER2, ER, and Survivin using qRT-PCR 24hrs post treatment and histological examination. *L. quinquestriatus* & *M. xanthina* venoms and tamoxifen had IC_{50} at 2.5, 34.4 & 27.8µg/mL with *MCF-7* cell line respectively. Cell cycle was ceased at G1/S phase of *L. quinquestriatus* and *M. xanthina* compared to tamoxifen. In gene expression analysis, venoms and tamoxifen exhibited apoptotic effect against *MCF-7* cell lines by elevated expression of Caspase3 & depletion of HER2, ER, and Survivin. Histological examination showed apoptotic and slightly necrotic features in both venoms. Tamoxifen induced a number of apoptotic bodies and colonies of necrotic cells

Keywords: Venom, *Montivipera xanthina*, *Leiurus quinquestriatus*, *MCF-7* breast cancer cell line, cytotoxicity, apoptosis, Caspase-3, HER2, ER, Survivin.

Introduction

Cancer is a group of diseases causing uncontrolled growth of abnormal cells characterized by insufficient apoptosis and metastatic capabilities (American Cancer Society, 2017). Breast cancer is one of the commonest diseases in women (lobules, ducts & connective tissues) and the second cause of their mortality (Bajrami *et al*, 2018). Globally, breast cancer was estimated with 1.67 million new cancer cases; 11% of all diagnosed cancers (Feraly *et al*, 2015). Sporadic breast cancer (somatic genes related-mutations combined with environmental factors) and/ or hereditary cancer (Kenemans *et al*, 2004). The naturally extractions were attractive alternative drugs. Bioactive molecules in venoms extracts of several animals such as, snakes, scorpions, spiders, bees, and frogs stimulated new pharmacological anticancer agents (Abdel-Aziz *et al*, 2017). The venoms consisted of mixtures of proteins, non-proteins, peptides, enzymes and toxins intended to target vital biological and physiological actions (Gomes *et al*, 2010). Bioactivity and biodiversity of venoms components were characterized among highest specific anticancer agents safe to normal cells and tissues (Omran, 2003). Snake and scorpion venoms proved effective anti-cancer therapy (Chaisakul *et al*, 2016). Snake venom, mixture of bioactive peptides, proteins, enzymes and toxins include cytotoxins, cardiotoxins & neurotoxins, exhibited cytotoxic activities (Aart and Khusro, 2013). Venom targets cellular metabolism alterations rendering it potential anticancer & anti-oxidant to block some specific ion channels, inhibited angiogenesis, obliterated metastasis, & activated intracellular pathways causing apoptosis (Ebrahim *et al*, 2015). Snake venom antitumor activity: phospholipase-A_2 from *Bothrops jaracaussu*, nigexine from *Naja nigricollis*, L-amino acid oxidase from *Vipera berus*, contortrostatin from *Agkistrodon contortrix* and viperistatin from *Vipera xanthina* (Bonfim *et al*, 2009). Consequences of scorpion stings on affected victims, including man, were known from ancient times, peptides employed action basis on affecting specific

ion channels function (Na^+, K^+, Ca^{++} & Cl^-) active alteration of abnormal expression binding cancer process included cell adhesion, invasive proliferation, migration and apoptosis (Ortiz and Possani, 2018). *In vitro* & *in vivo* studies proved anticancer potential bioactive components of scorpion venom: bengalin from *Heterometrus bengalensis*, margatoxin from *Centruroides margaritatus*, neopladine 1 & 2 from *Tityus discrepans* and chlorotoxin & charybdotoxin from *Leiurus quinquestriatus* (Ding *et al*, 2014). HER-2, Estrogen receptor, Caspase-3, and Survivin are proteins with a pivotal role in metastasis and apoptosis. Human epidermal growth factor receptor-2 (HER2) is a member of HER receptors family with a tyrosine kinase activity. HER2 has marked role in growth, survival, differentiation & proliferation of cells by many signal transduction pathways (Iqbal, 2014). Estrogen receptors are proteins actvated by estrogen hormone, proteins control gene expression & cells proliferation (Groos and Yee, 2002). Capasase-3, a thiol protease of aspartate cysteine protease family is one of executioner caspases, & a key mediator of apoptotic pathways (Devarajan *et al*, 2002; Donovan *et al*, 2003). Survivin, silenced in normal tissue but, overexpressed in malignant tissues consists of an inhibitor of apoptosis proteins with overexpression most cancer cells contributed to resistance to apoptotic stimuli and chemotherapies, thus contributed to on-going survival. It inhibits apoptosis promoting cell division, and enhance. Cross-correlation between genes was a significant determined drug-induced anti-proliferative, anti-antigenic, anti-metastatic, and apoptotic activities (Lv *et al*, 2010).

This study aimed to evaluate the anticancer potential of animal venoms of *Leiurus quinquestriatus* and *Montivipera xanthina* compared to synthetic hormonal anticancer drug (Tamoxifen®) on MCF-7 cells (human breast adenocarcinoma cell line).

Materials and Methods

Cell culture and MTT staining, penicillin-streptomycin, 25% trypsin-EDTA, and propidium iodide DNA staining were purchased (Sigma Chemicals Co, USA). Cell culture grade dimethyl-sulfoxide (DMSO), medium RPMI1640, human breast cancer (MCF-7) cell line was kindly supplied from R & D Sector, The International Center for Advanced Research. Venoms of *M. xanthina* and *L. quinquestriatus* and Tamoxifen® drug were purchased (VACSERA, Egypt). Annexin V-FITC was from Bio-Vision Research Products, USA & iScript™ One-Step Real Time-PCR Kit with SYBR® Green was from Bio-Rad Laboratories, USA. Venoms were prepared by diluting 1mg of each in 1mL phosphate buffer saline (PBS). Drug was prepared by dissolving 10mg of Tamoxifen tablet in 5mL DMSO solution. All materials were serially diluted in RPMI-1641 media to need concentrations (Gajski *et al*, 2014).

Maintenance of cell line and culture conditions: *MCF-7* (Human breast adenocarcinoma cell line) was provided by VACSERA. Cells were cultured in RPMI medium in the T-75 flasks (Griener, Germany) supplemented with 10% fetal bovine serum. Cultures were kept at 37°C, & 95% humidity with 5% CO_2. Monolayer cells were trypsinized once reached 80% confluency (Masters, 2000).

Cell counting: Accurate cell number in suspension was calculated using haemocytometer (Soliman *et al*, 2013). Double fold dilution of the original cell suspension was prepared by adding 0.5mL of undiluted cell to 0.5mL of 0.4% trypan blue dye, mixed well and immediately aspirated onto haemocytometer counting chambers.

Cytotoxicity using MTT reagent assay: Cells were put in the 96-well tissue culture plate with 100µL of RPMI medium. 200µL of the materials and diluted serially twice. Plates were then incubated with each venom concentrations for 24hr at 37ºC. For MTT assay, the 96-well plate was washed by PBS & 50µL MTT, added into each well, incubated for 3hr at 37ºC. 50µL of isopropanol was added and then incubated for 5min at 37ºC. Plates were read by ELISA reader at wavelength 570nm and absorbance correlat-

led with cell number (Houghton *et al*, 2007). Cytotoxic effects of venoms on the *MCF*-7 cell lines were expressed as IC_{50} value (drug reduced absorbance of treated cells by 50%). Inhibitory IC_{50} of compounds was assessed by Masterplex software (2010).

Inverted phase contrast microscopy: Morphological changes of treated MCF-7 cells with each venom and tamoxifen were examined 24hr post treatment (Soliman *et al*, 2013).

Cell cycle analysis using propidium iodide DNA staining by flow cytometry (Riccardi and Nicoletti, 2006): Cells were harvested in an appropriate manner, washed with PBS, and fixed in cold 70% ethanol and vortexing, pellet was added drop wise. Cells were fixed for 30min at 4^{o}C and minimized clumping. After fixation, cells were washed in PBS & spun at 850g. Supernatant was discarded and cells were treated with ribonuclease I (100mg/ml stock solution). 50µl of 100µg/ml sock of RNase were added to ensure DNA was stained. Propidium iodide (200µl) was added (50µg/ml stock solution).

Apoptotic detection using Annexin V-FITC assay by flow Cytometry: 1-5x10^{5} cells were collected by centrifugation, suspended in 500µl of 1X binding buffer. 5µl of Annexin V-FITC and 5µl of propidium iodide (PI 50mg/ml) were added. Cells were left at room temperature for 5min in dark. Annexin V-FITC binding was analyzed by flow cytometry (Ex =488 nm; Em= 530 nm) using FITC signal detector and PI staining by phycoerythrin emission signal detector. For cells adherent, they were gently trypsinized and washed once with serum-containing media before incubation with Annexin V-FITC.

Gene expression analysis after treatment with venoms and tamoxifen: Expression of regulatory and apoptotic genes (Her2, ER, Casp-3, & Survivin) were examined by real-time PCR in *MCF-7* cell lines. Primer sequences of oligonucleotides were used (Tab. 1). Total RNA was extracted from treated and untreated *MCF-7* cells 24hr post treatment using iScript™ One-Step real-time PCR Kit with SYBR® Green (Bio-Rad Laboratories, USA) by real-time quantitative-PCR of RNA templates. cDNA synthesis and PCR amplification were done simultaneously. Melting curve of PCR products was performed by StepOneTM Real Time Systems software V.2.2.2.

The relative quantification for the genes (HER2, ER, Casp-3, & Survivin) was calculated using equation: FLD= e^–[(TE-HE)-TC-HC] =e^-ΔΔCT

Table 1: Primer sequences for genes (Quality Endorsed Company)

HER2-F: 5'-ACAACCAAGTGAGGCAGG TC-3'
HER2-R: 5'-GTATTGTTCAGCGGGTCTCC-3'
ER-F 5'-TAC TGC ATC AGA TCC AAG GG-3'
ER-R 5'- ATC AA T GGT G CA CTG G TT GG -3'
Casp-3-F 5'-TTC ATT ATT CAG GCC TGC CGA GG-3'
Casp-3-R 5'-TTC TGA CAG G CC ATG TCA TCC TCA-3'
Survivin-F: 5'-GCA TGG GTG CCC CGA CGT TG-3'
Survivin-R: 5'-GCT CCG G CC AGA GGC CTC A A-3'
β-actin-F 5'-GTGACATCCACACCCAGAG G-3'
β-actin-R 5'-ACAGGATGTCAAAACTGCCC-3'

Histologically: Detached and adhered cells were collected post treatment using trypsin. Pelleted cells were re-suspended in PBS and a part (50µL) was dispensed on glass slide, dried and fixed using methanol. Fixed slides were rehydrated in descending ethanol and washed in distilled water for 5 min. Slides were immersed in filtered hematoxylin stain for 3 min, rinsed in distilled water twice, immersed in filtered eosin stain for 5 sec, and washed with distilled water. Clean slides were immersed in xylene, mounted in Canada balsam, covered cover-slips and left to dry.

Statistical analysis: Data were processed (Snedecor and Cochran, 1982); where minimum, maximum, mean value, standard deviation, standard error, and range were presented. For significance *t.* test was used for difference between two means,

Results

Table1: Inhibitory effect of *M. xanthina, L. quinquestriatus* venoms and Tamoxifen on MCF-7 cell line by MTT assay.

	Xanthina/*MCF-7*	Tamoxifen/*MCF-7*	*Leiurus*/*MCF-7*
IC_{50}	34.40	27.80	2.50

Cytotoxicity of *M. xanthine, L. quinquestriatus* venoms and Tamoxifen were 2.5µg /mL, 27.8µg/mL & 34.4µg/mL respectively on MCF-7 cell line using MTT assay (Fig. 1).

Table 2: Determination of cell cycle phases of *MCF-7* cell line treated with animal venoms and Tamoxifen using Propidium iodide DNA staining by Flow Cytometry.

	Sample (Conc. ug/ml)	Phases of cell cycle				
	Sample code	%G0-G1	%S	%G2-M	%Pre-G1	Comment
1	Tamox/*MCF-7*	43.11	20.97	2.87	33.05	PreG1apoptosis&Cell growth arrest@G1/S
2	*Leiu /MCF-7*	62.29	17.23	1.28	19.20	PreG1apoptosis&Cell growth arrest@G1/S
3	*Xan /MCF-7*	59.41	23.16	1.79	15.69	PreG1apoptosis&Cell growth arrest@G1/S
4	Cont.*MCF-7*	72.33	15.31	9.41	2.95	

Morphology showed cell rounded, condensed with cellular rupture and significant reduced treated cells number compared to control ($p \geq 0.05$). Treated *MCF-7* cells with venoms & tamoxifen were significant time-dependent increased cells in G0/ G1 population, followed by cells arrest in G1/S after 24hr. no apoptosis in control (Fig. 3-4).

Table3: Death of treated *MCF-7* cells with animal venoms and tamoxifen drug in total, early & late apoptosis and necrosis.

Variants	Apoptosis total	Apoptosis early	Apoptosis late	Necrosis
Tamo/*MCF-7*	33.05	9.66	20.14	3.25
Leiu /MCF-7	19.20	6.41	8.16	4.63
Xan /MCF-7	15.69	5.46	7.88	2.35
Cont.MCF-7	2.95	1.38	0.52	1.05

Venoms and Tamoxifen induced apoptosis on *MCF-7* cell line, in total, early, & late apoptosis and necrosis by venoms and tamoxifen compared to control (Fig. 5).

Table4: Gene expression levels of HER2, ER, Survivin and Casp3 in treated and untreated MCF-7 cell line.

Ser	Sample code	HER2	ER	Survivin	Casp3
1	Tamoxifen/*MCF7*	0.064824	0.545211	0.648558	8.392726
2	*Leiurus /MCF7*	0.077763	0.476295	0.520668	6.612089
3	*Xanthina/MCF7*	0.080746	0.404457	0.450689	4.771106
4	cont.*MCF7*	1	1	1	1

Gene expression levels of pro-apoptotic genes as Caspase3 and anti-apoptotic genes as HER2, ER & Survivin were examined in MCF-7 cell line post treatment with IC_{50} of venoms and tamoxifen compared to control. Venoms and tamoxifen induced the up regulation of pro-apoptotic gene Caspase3 and down regulation of anti-apoptotic genes (HER2, ER & Survivin) on breast cancer *MCF-7* cell lines (Fig. 6).

Histologically treated *MCF-7* cell line with venoms & tamoxifen compared to untreated one were detected. Control showed regular tumour of cellular and nuclear pleomorphisms. Cells were regular, hyperchromatic and condensed nuclei. Cellular outline regular without any folding in cellular membrane. Some cells showed cellular and nuclear pleomorphism (Fig. 7). Treated *MCF-7* cells with tamoxifen showed late apoptosis of multiple bodies and remnants of necrotic cells (Fig. 8). *MCF-7* cells treated with *Leiurus* venom were shrunken with irregular nuclear & cell membranes, membrane blebbing, peripheral condensation of chromatin and nucleolar segregation. Swollen necrotic cells with irregular membrane, necrotic cell debris and apoptotic bodies (Fig. 9). *MCF-7* cells treated with *Montivipera* venom showed apoptosis as shrunken cells, nuclei irregular, cellular & nuclear membranes, peripheral condensation of chromatin and nucleolar segregation (Fig. 10).

Discussion

Chaubey (2017) reported a significant advancement in cancer therapy possibility to

synthesize peptides and proteins specific for oncoproteins. Chaisakul *et al.* (2016) found that the anticancer effects of venoms of animal species including snakes and scorpions and their biochemical derivatives proved to be potential therapeutic tools. Cytotoxicity of snake families and their potential induced anticancer activities in sarcoma, leukemia and carcinoma models. Venom from *Viperdae* family acts directly affected breast cancer cells (Gomes *et al*, 2010). Yalcin *et al*. (2014) reported that *M. xanthina* venom was used for different cancer cells types as anticancer agents. Ahluwalia and Shah (2014) found that by increasing concentration of snake venom, cytotoxicity increased and affected proliferation of breast cancer cells. Inhibitory concentration of Viperidae snake venoms of *M. xanthina*, *Crotalus oreganus cerberus* and *Bothrops alternatus* toward *MCF-7* cells after 24hr incubation was at 4.2, 17.5 & 63.5µg/mL respectively (Yalcin *et al*, 2014; Bradshaw *et al*, 2016). IC_{50} for Russell's viper venom was 35.5µg/mL on leukemic cancer cells after 48hr post treatment (Gomes *et al*, 2015).

In the present study, *MCF-7* cells treated with *Montivipera* venom for 24hr, IC_{50} value was 34.4µg/mL. This agreed with Gomes *et al*. (2015) but disagreed with Yalcin *et al*. (2014) and Bradshaw *et al*. (2016). This might be due to difference in animal species and/or habits as venom source. Lethality of *Leiurus* venom (17mg) injected into normal man caused less than 50% survival chance, and reduced cell survival chance at higher concentrations and destruction of other cells by different mechanisms. Abdel-Aziz *et al.* (2017) reported that IC_{50} of *Leiurus* venom was 2.5µg/mL on *MCF-7* cell line after 48hr incubation. In the present study, the IC_{50} of *Leiurus* on *MCF-7* cell line after 24hr incubation was 2.50µg/mL. But, MCF-7 cell line exposed to tamoxifen for 72hr, IC_{50} was 23.9µg/mL (Lord and Ashworth, 2010). In the present study, IC_{50} of *MCF-7* treated with tamoxifen was 27.8µg/ mL. Difference in both results was quite similar with slight variation due to artefacts and minor data anomalies. Scorpion venom has higher toxins than snake one (Omran, 2003). Venom of *Leiurus* was more toxic and potent than that of *Montivipera* and tamoxifen. Snake venoms reduced cell proliferation and induced morphological alterations related apoptosis: detachment of cells, rounding, chromatin condensation, cytoplasmic blebs and irregularly shaped (Ebrahim *et al*, 2014). *Montivipera* venom by inverted microscopy showed changes in *MCF-7* cell line (Yalcin *et al*, 2014). Calderon *et al.* (2014) found that lectins isolated from *Vipera* venom affected proliferation of human cancer cell lines and alter cell adhesion inducing apoptosis. Also, scorpion venom inhibited growth and proliferation in cancer cell types (Aarti and Khusro, 2013). *Leiurus* venom caused morphological changes, exhibited apoptotic cells as, condensation and compartmentalization of nuclear and cytoplasmic materials (Omran, 2003). Chaisakul *et al*. (2016) isolated Chlorotoxin (CITx) from *L. quinquestriatus* venom conjugated with its synthetic compound (TM601) inhibited cancer development by attenuating Cl^- conductance & angiogenesis and CTLx prevented aggressive metastatic breast cancer cells. Treatment of breast cancer with tamoxifen gave effective palliation but without eradication of cancer cells. It caused cytostasis in *MCF-7* cells by proliferation of cell line and morphologically tamoxifen analogue toremifene induced apoptosis (60%) of *MCF-7* cells (Wilson *et al*, 1995). Venoms and tamoxifen on *MCF-7* cell line gave significant inhibitory effects, cellular proliferation and morphological changes related to cell death as condensed in size and a small round cell shape after 24hr incubation significantly reduced cell number. Reduction of cell proliferation might be due to lectins in venom of snake or CTLx or scorpion. Chaisakul *et al.* (2017) showed that cytotoxins of snake venom effected cancer cell proliferation, migration, invasion, neovascularization and apoptosis by arresting cell cycle in Pre-G1 population.

Russell's viper venom arrested cell cycle in G1/S phase on leukemic U937 cancer cells 24hr post treatment (Gomes *et al,* 2015). *Salmosin disintegrins* from Korean snake inhibited angiogenesis without toxicity to normal blood vessels and induced cell cycle arrest led to apoptosis (Chaisakul *et al,* 2016). Obtustatin disintegrins from *Vipera lebetina obtuse* inhibited angiogenesis reduced tumour development and hindered growth (Rahman and Choudhary, 2016). Gomes *et al.* (2010) found that charybdotoxin (CTX) of *L. quinquestriatus* venom caused a slight depolarization in breast cancer cells, arrested cell cycle at early G1, late G1, & S phases and gathered cells in S phase by blocking Ca^{2+}activated K^+ channel. Blockage of specific ion channels impaired cancer growth & metastasis and activated intracellular pathways to cell cycle arrest & apoptosis (Chaubey, 2017). Tamoxifen inhibited proliferation of *MCF-7* cells and reduced in S phase, accumulation in G1 early phase, concomitant depletion of G2/M phase by hindering cell cycle in early G1 phase (Osborne *et al*, 1983). In the present study, both venoms and tamoxifen induced G1/S cell cycle arrest on *MCF-7* cells after 24hr incubation. Anti-proliferative activity inhibited cell growth in G1 /S causing apoptosis during Pre-G1 by targeting cancer cell proliferation, migration, invasion, neovascularization and apoptotic activity (Chaisakul *et al*, 2016). Cytotoxicity of snake v-nom targets cellular metabolism alterations and affected cancer cells; blocked some specific ion channels, inhibiting angiogenesis & activating intracellular pathways causing apoptosis (Ebrahim *et al*, 2015). They potentially induced apoptosis by forming apoptotic body, increasing in subG1 population, fragmenting cells DNA and cleaving poly (ADP-ribose) polymerase (PARP) (Gomes *et al,* 2010). Activity of Na^+, K^+, Ca^{++} & Cl^- ion channels altered abnormal expression binding to cancer processes involving cell volume and motility and cell proliferation with final death (Ding *et al,* 2014). Rahman and Choudhary (2016) reported that Viperistatin from *Vipera xanthina* venom was characterized as KTS motif in the integrin active binding site loop to block α1β1 integrin. Anti-angiogenic cyclic KTS peptides blocked binding of α1β1 & α2β1 integrins. Obtustatin from *V. lebetina obtuse* venom, activated inhibiting proliferation, & alteration in cell adhesion by apoptosis induction (Calderon *et al,* 2014). Chaisakul *et al.* (2016) reported that active components of snake venom as PLA2 and lectin from *Bothrops jararacussu* potential affected cancer. PLA2 induced apoptosis in cancers: breast adenocarcinoma, leukemia T and Erlich ascetic tumour cell line. Omran (2003) reported that apoptosis included a cascade of cytoplasmic and nuclear events causing a series of morphological changes and eventually cell demise by various stimuli. Nevertheless, necrosis caused cell lysis and extensive damage surrounding tissues physically and/ or oxygen starvation. Rapid cytotoxicity effects of *leiurus* venom occurred within 30 min. causing lysis of plasmalemma and organelle membranes that led to rapid death of a great cell population. Effects of cell death in *T47D* (breast cancer cell line) treated with *Leiurus* venom showed significant cell death in different concentrations (Omran, 2003). So, when chlorotoxin bind with Cl^- channel in membrane protein, chlorotoxin interacted and killed the cancer cells by ion modulation. Also, chlorotoxin bind with MMP-2 caused reduction of expression of MMP-2 and regression induction of aggressive metastatic breast cancer cells (King, 2011). But, tamoxifen and its analogues induced death of human breast cancer cells in both *in vivo* and *in vitro* studies. Tamoxifen and its metabolite 4-hydroxy caused morphological changes by cleavage of DNA inducing apoptosis on *MCF-7* cells (Wilson *et al*, 1995). In the present study, *MCF-7* cell line treated with venoms and tamoxifen showed cell death by using annexin V-FITC after 24 hr treatment. Apoptosis of treated MCF-7 with tested materials detected in total, early, and late apoptosis and necrosis as well. This agreed with

Shirazi *et al*. (2014) who found that *MCF-7* cell lines treated with Cobra venom gave early and late apoptosis and necrosis. This agreed with Omran (2003) who found that *Leiurus* venom caused an early significant apoptosis rather than necrosis on cancer cells. Apoptosis mediated by toxicity caused cell damage such as swelling, rupture, and necrosis. Tamoxifen treated *MCF-7* cell line did not develop apoptosis under same conditions (Wilson *et al*, 1995), but induced apoptosis on *MCF-7* cells. Venoms altered gene expression and increased expression of pro-apoptotic proteins (Chaisakul *et al*, 2016). Snake toxins increased expression of pro-apoptotic proteins and induced apoptosis via ROS-in-dependent mitochondrial dysfunction pathway and caspase-dependant mechanism of Bax/Bcl-2 ratio (Ebrahim *et al*, 2015). Snake cytotoxins mediated apoptosis via cleaving poly (ADP-ribose) polymerase (PARP) (Gomes *et al*, 2010). LAAOs stimulated apoptotic activity by oxidative reaction mediated with released H_2O_2 led to trigger apoptosis (Gomes *et al*, 2010). Chaisakul *et al*. (2016) isolated LAAO from king cobra venom exhibited a specific anticancer activity on human breast adenocarcinoma cell line by extrinsic and intrinsic pathways enhanced of activities of caspase-8 & caspase-9 induced cell apoptosis. Induction of cell-cycle arrest in breast cancer cell lines was done by caspase-dependent apoptosis by snake venoms. The expression of active Caspase-3 was increased in *MDA-MB-231* and *MCF-7* breast cancer cells treated with snake venom (Al-Sadoon *et al*, 2018). Scorpion venom induced anti-proliferative effect by arresting S-phase and apoptosis through Capase-3 activity, nitric oxide production, DNA fragmentation & depolarization of mitochondrial membrane (Ahluwalia and Shah, 2014). Chaisakul *et al.* (2016) found that bengalin from *Heterometrus bengalensis* venom acted on leukemic cells by activating Caspase-3, Caspase-9 and cleavage of induced poly (ADP-ribose) polymerase (PARP) reduced cell proliferation causing apoptosis. Activation of Caspase-3, Caspase-7, Caspase-8, and Caspase-9 occurred after breast cancer cells tamoxifen treatment (Moriai *et al*, 2008). Tamoxifen induced potential activity of Caspase-3 in ER-negative breast cancer cell mediated by cleavage of fluorogenic tetra peptide substrate and poly (ADP-ribose) polymerase (Mandlekar *et al*, 2000).

In the present study, gene expression levels of pro-apoptotic genes such as Caspase3 on *MCF-7* cell line post treatment with IC_{50} of venoms and tamoxifen occurred in treated MCF-7 with tested materials. The present study reported the up-regulation of Capsase-3 gene in breast cancer cell lines treated with venoms and tamoxifen. Snake venom inhibited migration of HER2 positive breast cancer cells by interrupting signal from epidermal growth factor receptors, applied stronger cytotoxic effects than therapies targeting receptors only (Karem *et al*, 2017). Ma *et al*. (2017) found that ADAM proteins and matrix meta-lloproteinases from scorpion venom activated HER2 receptor targeted for breast cancer cells. Proteolytic enzymes: Jararhagin & Jararhagin C from *Bothrops jararaca* act by cleavage of growth factors and corresponding receptor (HER2) causing degradation of extracellular matrix & inheritance of growth signalling. Overexpression of HER2 was associated in tamoxifen resistance in human breast cancer cells due to sensitivity (Shou *et al*, 2004). There existed ER-α36-EGFR/HER2 positive regulatory loops in HER2-expressing breast cancer cells and disruption of these regulatory loops restored tamoxifen sensitivity in these cells. (Yin *et al*, 2015). The present study showed expression level of anti-apoptotic gene of HER2 in *MCF-7* cell line post treatment with IC_{50} of venoms and tamoxifen. Down-regulation of HER2 gene was detected post treatment. The present results and others proved down-regulation of HER2 gene in breast cancer cell lines treated with venoms. But, not in tamoxifen treated *MCF-7* cell line that might be attributed to difference between HER2 expression level and its' ac-

tion. Purified snake venoms showed cytotoxic activity against *MCF-7* (ER+) & *MDA-MB-231*(ER-) cells in a dose dependent manner (Attarde and Pandit, 2017). Erdes *et al*. (2014) found that *L. abdullah bayrami* venom has an anti-proliferative effect on *MCF-7* (ER+) breast cancer cell and *L. quinquestriatus* venom has anticancer effect on *MCF-7* cell line (Abdel-Aziz *et al*, 2017), venom exhibited broad range of anticancer activity on *MDA‑MB‑231* (ER-) cell line (Al-Asmari *et al*, 2015). Tamoxifen had influence on cytoskeleton of two ER positive cell lines as the standard therapy with ER+ breast cancer. But, many patients developed resistance to tamoxifen and reflected to both ER+ & ER− cell lines (Rondon-Lagos *et al*, 2016). Down-regulation of anti-apoptotic gene ER was post treatment. The present study and others reported down-regulation of ER gene in breast cancer cell lines treated with venoms and tamoxifen but with higher probability of resistance to tamoxifen. One of the prominent genes in P53 pathway is Survivin (BIRC5). Clinically, EME1, RAD51, EXO1, BLM expressions correlated with BIRC5 (coding for Survivin) and of prognostic value. Actively, Survivin depletion triggers P53 activation & sensitized cancer cells to of PARP inhibition (Véquaud *et al*, 2016). Inhibition of Stat3 decreased expression of Survivin in breast cancer cells. Scorpion venom decreased Stat3 on breast cancer lines, thereby decreasing expression of anti-apoptotic gene (Gritsko *et al*, 2006; Al-Asmari *et al*, 2018). But, tamoxifen induced apoptosis was blocked by Caspases present in breast cancer cells. Up-regulation of anti-apoptotic Survivin gene induced inhibition of Caspase-3 & Caspase-7. Down-regulation of Survivin stimulated tamoxifen induced apoptosis by introducing siRNA targeting Survivin gene in *MCF-7* cells. siRNA targeted-Survivin transfection did not induce apoptosis without tamoxifen treatment but augmented by induced apoptosis. Survivin acts as a factor conferred resistance against tamoxifen-enhanced apoptosis (Moriai *et al*, 2009). This study showed the gene expression level of anti-apoptotic gene of Survivin in *MCF-7* cell line post treatment with IC_{50} of venoms and tamoxifen. Down-regulation of anti-apoptotic gene Survivin occurred post treatment. Expression of anti-apoptotic Survivin gene reduced breast cancer cell line by induction of P53 pathway and/or inhibition of Stat3 signalling protein mediated by venoms. But, tamoxifen negatively regulated between Survivn gene and wild type Caspase-3 gene. Lipps (1998) reported that certain fractions isolated from snake venoms gave direct cytolytic activity on tumor cells. Snake cytotoxins induced apoptosis by forming apoptotic bodies and fragmented DNA cells (Gomes *et al*, 2010).

Venom post treatment was tissue necrosis, displayed potent cytotoxic effect and apoptotic effect on human leukemic cells (Ahluwalia and Shah, 2014). *Elapid* venom cytotoxins caused membrane damage and necrosis by mediating direct action with phospholipids membranes to form pores (Chaisakul *et al*, 2016). Omran *et al*. (2003) found morphologic dyed cells of scorpion venom were fragmented, condensed with shrinkage and vacuoles between adherent cells. By nuclear DNA fragmentation, venom caused apoptosis of tumour cells. *Odonotobuthus doriae* venom not only caused apoptosis but also inhibited DNA synthesis in *MCF-7* breast cancer cell line (Ahluwalia and Shah, 2014). Tamoxifen caused chromatin condensation, nuclear fragmentation & apoptosis (Mandlekar *et al*, 2000).

Conclusion

MCF-7 cells treated with venoms and tamoxifen histologically showed apoptosis as shrunken cell, shrunken nuclei, irregular cellular nuclear membranes, apoptotic bodies, peripheral ch-romatin condensation, nucleoli segregation and signs of necrosis. Snake (*Montivipera xanthina*) and scorpion (*Leiurus quinquestriatus*) venoms have anticancer action on human breast cancer compared with tamoxifen®. Anti-cancer was proved by venoms cytoxicity, cell cycle cytox-

icity, cell cycle arrest and up- and down-regulations of pro & anti-apoptotic genes.

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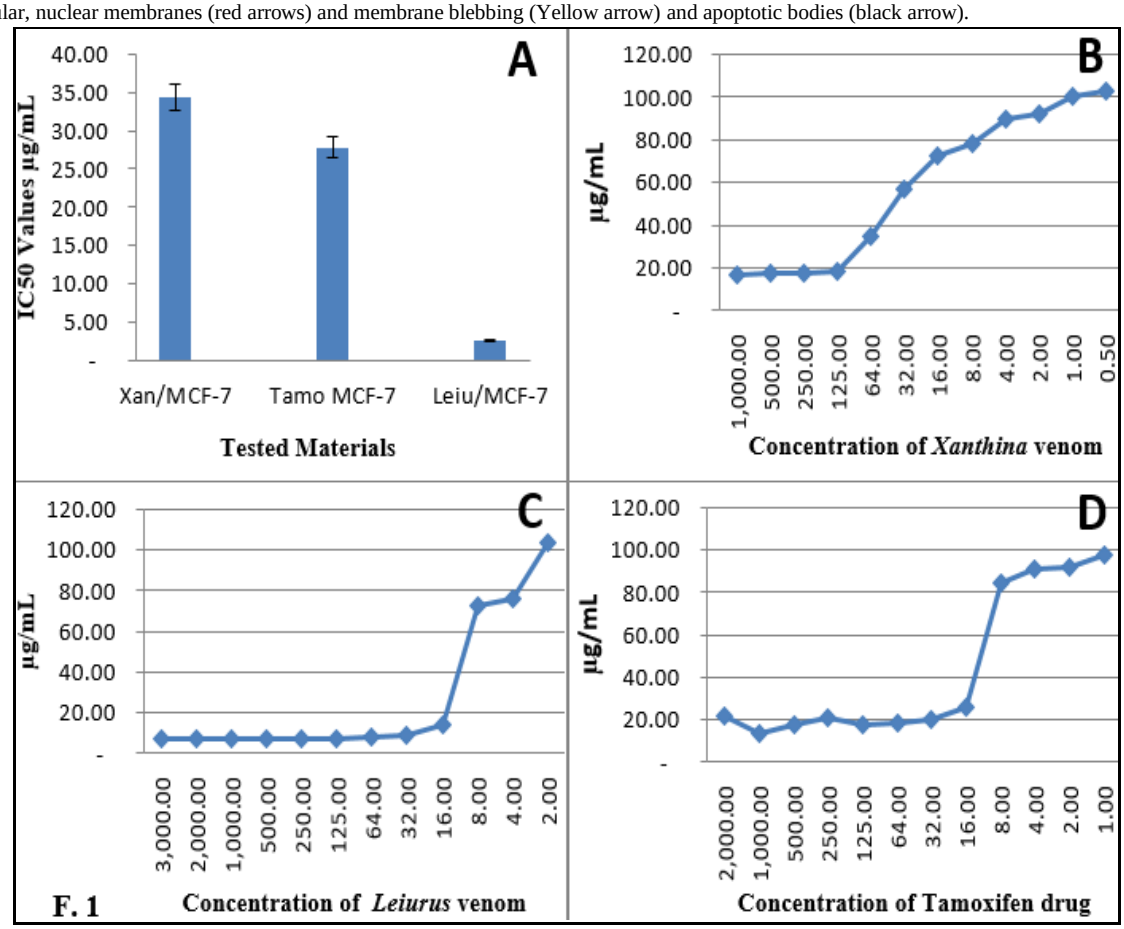
Explanation of Figures

Fig. 1: IC_{50} for *M. xanthina* & *L. quinquestriatus*, venoms and Tamoxifen on *MCF-7* cell line. (A) Venoms and Tamoxifen on *MCF-7* cell line. (B) *Leiurus* venom on *MCF-7* cell line. (C) IC_{50} of *Montivipera* venom on *MCF-7* cell line. (D) Tamoxifen on *MCF-7* cell line.
Fig. 2: Effects of venoms and tamoxifen on morphological alterations of MCF-7 showed induce anti-proliferative effects on treated *MCF-7*cell line compared to untreated one. (A) Untreated control *MCF-7* cells, (B) Treated *MCF-7* with 27.8ug/mL Tamoxifen, (C)Treated *MCF-7* with 34.4 ug/mL *Montivipera* venom, (D) Treated *MCF-7* with 2.5 ug/mL *Leiurus* venom.
Fig. 3-4: *MCF-7* Cell cycle treated with *Montivipera* and *Leiurus* venoms and tamoxifen.
Fig. 5: Apoptosis phases and necrosis of venoms and Tamoxifen on MCF-7 cell line. Apoptosis in total, early, and late and necrosis by *Montivipera* and Leiurus venoms and tamoxifen compared with control MCF-7 cells.
Fig. 6: Expression level of Pro and anti-apoptotic genes by R-T PCR in breast cancer cell linesMCF-7 treated with venoms and tamoxifen.
Fig. 7: Control cells showed regular tumor cells (Red arrow), Cellular and nuclear pleomorphisms (Green arrow).
Fig. 8: (A) Treated MCF-7 with tamoxifen showed apoptotic bodies (yellow arrows) and remnants of necrotic cells (black arrows). (B) Tamoxifen induced a number of apoptotic bodies (yellow arrows) and colonies of necrotic cells (Red arrow).
Fig. 9: (A) *MCF-7* treated with *Leiurus* venom showed shrunken cells with irregular nuclear and cell membranes (Red arrow), membrane blebbing (Green arrow), peripheral condensation of chromatin (Yellow arrow) and nucleolar segregation (Black arrow). (B) Treated cells with *Leiurus* detected swollen necrotic cells (Green arrows) with irregular cell membrane, necrotic cell debris (yellow arrows) and apoptotic bodies (Red arrows). (C) Treated cells showed shrunken with irregular nuclear and cell membranes (Red arrow), membrane blebbing (Green arrow), peripheral condensation of chromatin (Yellow arrow) and nucleolar segregation (Black arrow). (D) *Montivipera* venom on *MCF-7* induced small cells with irregular membranes (Red arrows), membrane blebbing (Black arrow) and apoptotic bodies (Green arrows). (E) *Leiurus* venom with *MCF-7* cell lines showed small cells with irregular nuclear and cell membranes (Yellow arrow), apoptotic bodies (Red arrow), and necrotic swollen cell mixed with euchromatin and heterochromatin (Black arrow).
Fig. 10: (A) *MCF-7* cells treated with *Leiurus* venom showed shrunken apoptotic cells (red arrows). (B) *MCF-7* cells with *Montivipera* venom showed small shrunken apoptotic cells with irregular cellular and nuclear membranes (Yellow arrows), peripheral condensation of chromatin (red arrows) as well as nucleolar segregation. (c) *Montivipera* venom on *MCF-7* cell lines; shrunken apoptotic cells with irregular cellular, nuclear membranes (red arrows) and membrane blebbing (Yellow arrow) and apoptotic bodies (black arrow).



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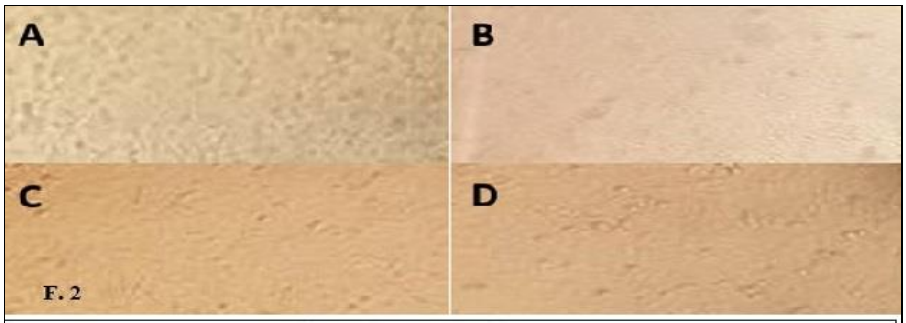

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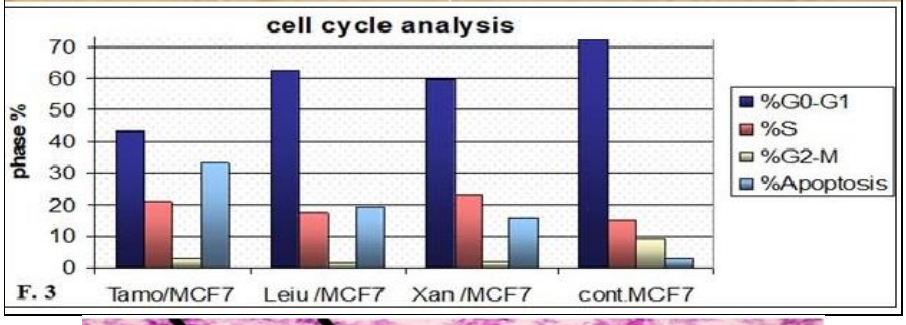

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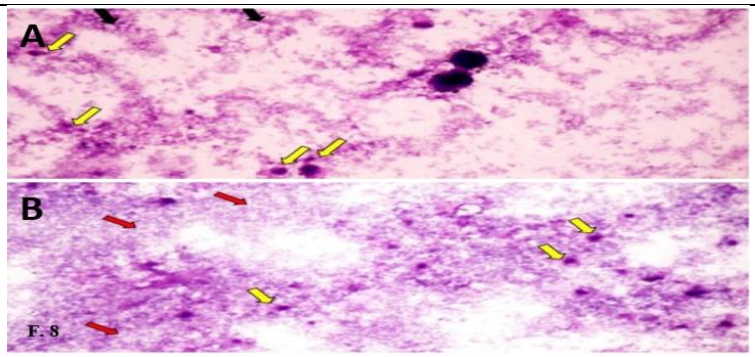

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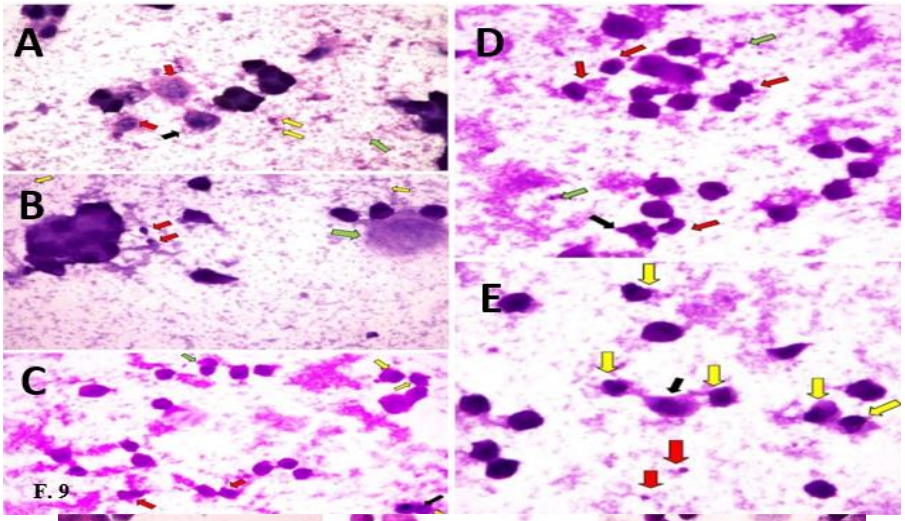

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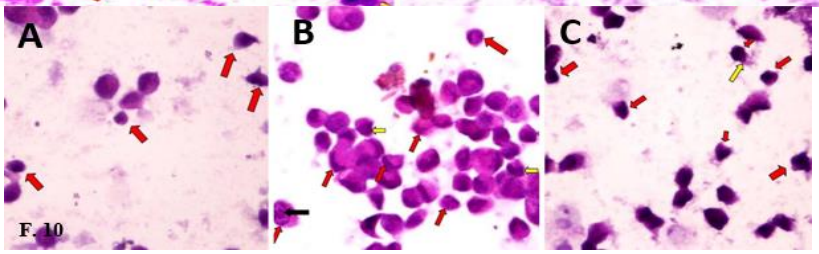

F. 10