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Biomolecular evaluation of apoptosis, cell cycle, oxidative stress, and limiting enzymes of the glycolytic pathway in hepatocellular carcinoma cell line HepG2 treated with crude snake venom with or without sorafenib

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Abstract

Background: Natural venoms have biological activities including anti-inflammatory, antimicrobial, and anticancer effects. Hepatocellular carcinoma (HCC) is still a worldwide problem and difficult to treat by chemotherapeutic agents especially sorafenib (SOR), as it evokes many harsh side effects and is disable to differentiate between normal and cancer cells. **Objective:** The present study aimed to test the hypothesis that combining crude venoms of the snake or the bee or the scorpion could synergistically enhance the antiproliferative effects of SOR in hepatocellular carcinoma cell line (HepG2). **Experimental design:** Separate crude venoms have been applied to HepG2 cells and normal human retinal cells (RBE1) for estimation of IC_{50}. The most effective venom has been combined with sorafenib in five nonconstant ratios and the combination index (CI) was estimated to expose their synergistic or antagonistic action. The best combination was used for downstream analysis. **Results:** The crude snake venom exhibited the most cytotoxic effect and the least IC_{50}. It has been combined with sorafenib, and the combination index (CI) was calculated. IC_{25} SV + IC_{10} SOR was the best combination with CI=0.209 indicating high synergistic cytotoxic activity against HepG2. The underlining molecular mechanisms of action, in terms of the expression level of apoptotic genes (p53, Bax, Caspase 3, and Bcl2), flow cytometric analysis of cell cycle, oxidative stress markers as well as the activity of some limiting enzymes in the glycolytic pathway (ALDOB, PK and LDH) have been investigated.

Conclusion: Our results suggest a novel synergistic, and anti-proliferative effect of snake venom with sorafenib on HepG2 cells.

Keywords: HCC, crude snake venom, sorafenib, cell cycle, oxidative stress, glycolytic pathway.

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1. Introduction

Cancer is a major public burden worldwide. It is a multi-genic and multi-cellular disease that can arise from all cell types and organs with a multi-factorial etiology **(Baskar et al., 2012)**. Hepatocellular carcinoma (HCC) is the cause of over 830,000 annual deaths and is expected to affect more than one million individuals by 2025 **(Llovet et al., 2021)**. In Egypt, it represents the fourth most common cancer **(Rashed et al., 2020)**. Different therapies have been used in cancer treatment, including chemo-, radio-, immuno-, and gene therapies **(Roy et al. 2017; Kumar et al. 2021)**.

There is a massive need to identify and develop novel compounds with the potential ability to treat one or more types of cancer, either individually or in combination with other approved therapeutic drugs **(Jaunky *et al*., 2016)**. On the other side, the search for cancer treatment from natural products is also a great demand to overcome the side effects as well as chemoresistance and to lower the cost of production. Sorafenib is a multikinase inhibitor used as systemic chemotherapy in patients with advanced-stage HCC and early-stage liver disease. It has been shown to improve overall survival, but with various severe side effects, while its cost is not negligible **(Ziogas and Tsoulfas, 2017)**. New therapeutic approaches are urgently needed for advanced or metastatic HCC. Over the years, animal venoms and toxins from several species such as snakes, bees, and scorpions have been widely studied for their potential as a major source of bioactive molecules **(Harvey, 2014)**.

Snake venom (SV) is a mixture of proteins with or without catalytic activity such as phospholipase A2, proteases, hyaluronidases, L-amino acid oxidases, acetylcholinesterases, growth factors, protein C activators, lectins, von Wille brand factor-binding proteins; peptides mainly comprising bradykinin potentiators and disintegrins; also it contains low molecular weight organic compounds (carbohydrates, serotonin, histamine, citrate, and nucleosides) and inorganic ions (calcium, cobalt, magnesium, copper, iron, and potassium) as well as enzymatic inhibitors **(Ramos and Selistre-De-Araujo, 2006)**. Snake venoms were reported to exhibit a cytotoxic effect against tumor cells **(Vyas *et al*., 2013)**. According to **Jain and Kumar (2012) and Vyas et al. (2013),** snake venom mediates its action by inhibiting nucleic acid synthesis, suppressing cell proliferation, decreasing the expression and activity of matrix metalloproteinases, inhibiting integrins, thereby preventing migration and invasion of cancer cells, and antiangiogenesis. Various active compounds obtained from snake venoms are cytotoxic by causing changes in the cellular metabolism, rate of apoptosis, cell cycle arrest which turns out to be fatal for cancer cells **(Santos et al., 2008 and Mahfouz et al., 2021)**.

On the other side, bee venom (BV) contains a variety of different peptides, including melittin, phospholipase A2, apamin, ado-lapin, and mast cell-degranulating peptide (MCDP); it also contains non-peptide components including lipids, carbohydrates, and free amino acids, all having many cellular activities **(Lariviere and Melzack, 1996)**. **Park et al. (2011)** demonstrated that either bee venom or melittin has anti-proliferative effects on various cancer cells such as prostate, liver, breast, cervical, and renal cancer cells through intrinsic or extrinsic apoptosis. According to **Soliman et al. (2019) and Mansour et al. (2021)**, melittin exerts a cytolytic activity and induces several cell death-related pathways such as apoptosis, inhibition of proliferation or angiogenesis, cell cycle arrest, and inhibition of cancer motility, migration, metastasis, and invasion.

Moreover, scorpion venom (SCV) has the property of inhibiting the growth of various types of cancers **(Liu et al., 2002)**. According to **Al-Asmari et al. (2018)**, the mode of action of scorpion venom includes the generation of ROS which causes DNA damage, leading to the upregulation of proapoptotic genes and

downregulation of antiapoptotic genes, initiating apoptosis in breast and colorectal cancer cell lines.

Given the above information, the present study was designed to investigate the antiproliferative activity and cytotoxic effects of crude venoms of the snake **(***Cerastes cerastes***),** the bee (*Apis mellifera lamarckii*) and the scorpion (*Androctonus australis)* from the Egyptian fauna against human hepatocellular carcinoma cell line (HepG2) being compared with the effect on normal human retinal cells (RBE1). The cytotoxic effects were evaluated by in vitro MTT assay in comparison with sorafenib as a positive control. The most effective venom has been combined with sorafenib in five nonconstant ratios and the combination index (CI) was estimated to expose their synergistic or antagonistic action and to test the feasibility of using the crude venom as a potent adjuvant with sorafenib in treating hepatocellular carcinoma. The best combination was used for downstream analysis of apoptosis, cell cycle, oxidative stress, and glycolytic pathway.

2. Material and Methods

2.1. Crude Venoms

Crude snake *(Cerastes cerastes*), bee (Apis mellifera lamarckii), and scorpion (*Androctonus australis)* venoms were purchased from VACSERA, Giza, Egypt.

2.2. Chemotherapy

Sorafenib (SOR) was a product of Hut Chem, China catno.284461-73-0.

2.3. Cell lines

Human hepatocarcinoma cells line HepG2 and RBE1 were obtained from Karolinska Institute, Department of Oncology and Pathology, Stockholm, Sweden.

2.4. Cell culturing

HepG2 cells were maintained in RPMI medium (Gibco, Gergy Pouloisa, France), while RPE1cells were maintained in DMEM medium (Dulbecco's Modified Eagle's Medium, Lonza, Basel, Switzerland). Media were applied to cells after being supplemented with 10% FBS. Cells were incubated at 37ºC in 5% CO_2 and 95% humidity. Subculturing has been done using Trypsin-Versene 0.15% (Lonza, Basel, Switzerland).

2.5. Cytotoxic assay

Cytotoxic effects were tested against HepG2 and RPE1 by in vitro MTT assay. Cells were counted by hemocytometer after being stained with trypan blue in 96-well plates and seeded in 1×10^4 /well. Different concentrations of the crude venoms (100, 50, 25, 12.5, 6.25, and 3.125 µg/ml) were applied in triplicates. The cells were incubated for 48 hours. Sorafenib was used as a positive control and 0.5% DMSO was used as a negative control**.** The media were discarded and 40 µl of MTT (Bio Basic, New York, USA) was added, then the plates were incubated for 4 h. The formed formazan crystals were solubilized by 100 µl of 10% sodium dodecyl sulfate (SDS) (ADWIC, Egypt) for 30 min at 37ºC in a 5% CO_2 incubator. The final violet color formed was then measured at 595 nm, on a microplate reader (StatFax-2100, Awareness Technology, Inc. USA). The concentration of the crude SV, BV, SCV, and SOR inhibiting 50% of cells (IC_{50}) was calculated, and the dose-response curve was plotted using the Graph Pad Prism software, version 8.

2.6. Determination of Combination Index

To quantitatively evaluate whether the combination of crude snake venom (SV) with sorafenib (SOR) might cause synergistic or antagonistic cytotoxic effects, the combination index (CI) was calculated using CompuSyn software, version 1. All experiments were run in triplicates in 2 independent experiments. Five combined treatments were prepared in non-constant ratios from fractions of IC_{50} of crude SV and SOR and applied to HepG2 and RPE1. The best combination with (a) the lowest SOR dose, (b) the highest cell death

on HepG2 cells and (c) the lowest cell death on RPE1 was selected for further downstream analysis.

2.7. Downstream Analysis

2.7.1. Gene expression by qRT-PCR

Total RNA was isolated from treated and untreated cells using a GeneJET RNA Purification Kit (Thermo Scientific, USA). RNA concentration and purity were determined by Nanodrop (Q5000, Quawell, USA) and 1% gel electrophoresis. RNA (5µg) was reverse transcribed using Quantiscript reverse transcriptase. The produced cDNA was used as a template to determine the relative expression of p53, Bax, Bcl2, and Caspase 3 genes in the presence of Syber green master mix using the Step One Plus real-time PCR system (Applied Biosystem, USA). The specific primers for amplified genes are described in Table (1). Each sample was normalized to the reference housekeeping gene GAPDH and untreated control. The relative level of transcripts was quantified by the thermal cycling conditions, melting curves temperatures, and calculation of relative expression using comparative analysis (2−ΔΔCt).

Table (1): Primers used for RT-PCR.

p53 gene F: 5′-ATGTTTTGCCAACTGGCCAAG-3′ **R: 5′-TGAGCAGCGCTCATGGTG-3′**
Bax gene F: 5′-GTTTCATCCAGGATCGAGCAG-3′ **R: 5′-CATCTTCTTCCAGATGGTGA-3′**
Casp3 gene F: 5′-TGTTTGTGTGCTTCTGAGCC-3′ **R: 5′-CACGCCATGTCATCATCAAC-3′**
Bcl-2 gene F: 5′-TTGTGGCCTTCTTTGAGTTCGGTG–3′ **R: 5′-GGTGCCGGTTCAGGTACTCAGTCA-3′**

2.7.2. Determination of oxidants/antioxidants status

Malondialdehyde (MDA) level and activities of superoxide dismutase activity (SOD), catalase (CAT), glutathione-S-transferase (GST), and reduced glutathione (GSH) were measured colorimetrically in HepG2 cells after homogenization using a commercial kit (Bio-diagnostic, Cairo, Egypt).

2.7.3. Cell cycle analysis using flow cytometry

Following trypsinization, HepG2 cells were centrifuged at 4500 rpm for 5 min, washed twice, resuspended in warm PBS, fixed by ice-cold absolute ethanol, and then incubated at −20°C for 24 h. Cells were re-suspended in propidium iodide (PI) solution containing 100 µl (0.02 mg/ml) PI, 50 µl (0.2 mg/ml) RNase A, and 0.1% v/v Triton X-100 in PBS, incubated in darkness for 30–60 min at room temperature, and then analyzed using Attune flow cytometer (Applied Bio-system, USA).

2.7.4. Evaluation of intracellular limiting enzymes of the glycolytic pathway and ALDH1A1-detoxifying enzyme

HepG2 cells were incubated with the best combination of SV and SOR for 48 h, then collected in a Falcon centrifuge tube and the supernatant was discarded. The activities of glycolytic enzymes PK, ALDOB, LDH, and the detoxification enzyme ALDH1A1 were evaluated using the simple step ELISA Kits according

to the manufacturer's instruction and read on ROBONIK P2000 Eliza Reader at the specific wavelength.

2.8. Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM). Data were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Tukey Kramer multiple comparisons using Graph Pad Prism software version 8. Significant differences among means were estimated at $p < 0.05$.

3. Results

3.1. Cytotoxic effect and IC_{50} of individual crude venoms and sorafenib

Data presented in Fig.(1a-d) revealed that among the three selected crude venoms, SV exhibited the highest cytotoxic effect on HepG2 cells with IC_{50}= 5.46 µg/ml versus7.94 µg/ml on the RPE1 cells, while SOR showed high cytotoxic effect with IC_{50}=12.58 and 5.88 µg/ml on HepG2 and RPE1 respectively. These data indicate the great sensitivity of both liver cancer and normal cells to SV and SOR. Accordingly, crude SV has been selected to study its combined cytotoxic effect with SOR in five non-constant ratios for clarification of their synergistic or antagonistic action.

3.2. Cytotoxic effect and IC_{50} of combined crude SV and SOR

As presented in Table (2), the five selected non-constant ratios showed different cytotoxic effects and combination index (CI) on both HepG2 and RPE1. CI < 1 indicates synergistic action while CI >1 indicates antagonistic action. The best synergistic cytotoxic effect (100% inhibition) was achieved by a combination of 1:1 or 1:1/2 (SV+SOR) on HepG2 cells with CI= 0.038. Meanwhile, both combinations showed synergistic inhibitory effects on normal cells with 97.8% inhibition (CI= 0.058) and 81.5 % inhibition (CI= 0.24), respectively. On the other hand, the fifth combination (1/2:1/5) showed the least cytotoxic effect (80.2%) on HepG2 cells with CI= 0.209. Meanwhile, it showed the best antagonistic action (CI=12.81) with the least inhibitory effect on normal cells (10.13%). This is why the fifth combination was selected for downstream analysis, being considered the safest combination on normal cells.

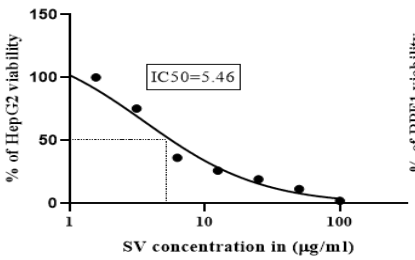
Table (2): Effect of different combinations of IC50 of both crude snake venom (SV) and sorafenib (SOR) on cytotoxicity as measured by combination index (CI).

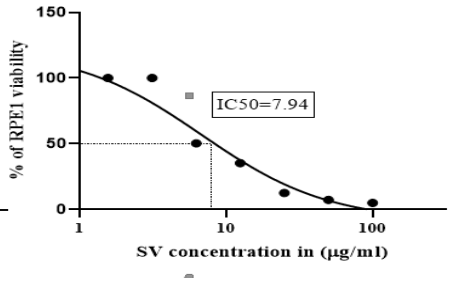
	Combinations		SV+SOR (HepG2)				SV+SOR (RPE1)	
No	**Ratio of IC50 SV: SOR**	**Dose in (µg/ml)**	**%Cell Death**	**CI**	**No**	**Dose in (µg/ml)**	**%Cell Death**	**CI**
1	1:1	5.4+12.58	100%	0.038	1	5.4+12.58	97.8%	0.058
2	1:1/2	5.4+6.29	100%	0.038	2	5.4+6.29	81.5%	0.24
3	1:1/5	5.4+2.51	97%	0.057	3	5.4+2.51	67.8%	0.675
4	1/2:1/2	2.7+6.29	93.8%	0.137	4	2.7+6.29	38.2%	1.39
5	**1/2:1/5**	**2.7+2.51**	**80.2%**	**0.209**	**5**	**2.7+2.51**	**10.13%**	**12.81**

* CI <1 = synergism
** CI >1 = antagonism

Fig. (1a-d): Cytotoxic effect and IC_{50} (µg/ml) of different crude venoms and sorafenib (SOR) against human hepatocellular carcinoma (HepG2) and human retinal cells (RPE1) after 48h of treatment.

(a)





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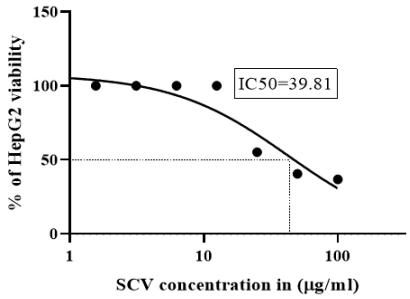
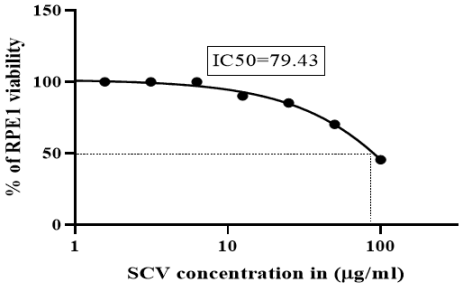
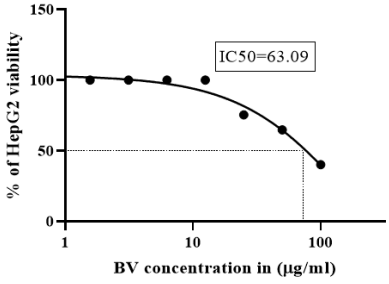
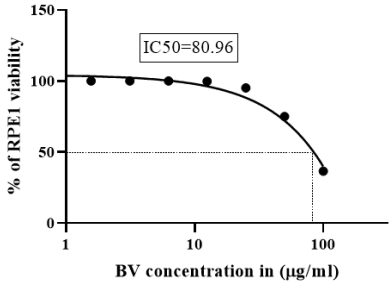


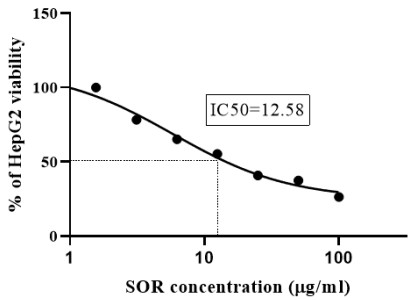


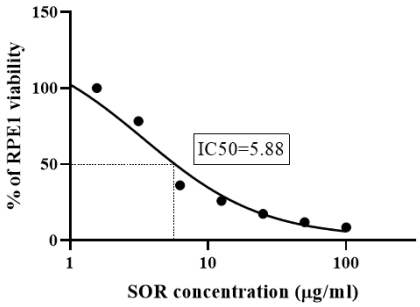
(c)





(d)





Effect of SV, SOR, and their combination on oxidative stress

Data illustrated in Fig. (2a-e) revealed a significant elevation in the level of MDA, but a significant reduction in the level of CAT, GSH, and GST in different treated groups as compared to the control untreated group ($P \leq 0.05$). The combination of SV-SOR showed the highest MDA value and the least CAT, GSH, and GST indicating stimulation of oxidative stress. On the other side, SOD showed a significant elevation under single and combined effects of SV and SOR with the best effect being encountered under their combination indicating some type of resistance to oxidative stress.

3.3. Effect of SV, SOR, and their combination on gene expression of apoptotic markers

As demonstrated in Fig. (3a-d) gene expression levels, measured by fold change of the proapoptotic genes (p53, Bax, Cas3) showed significant up-regulation versus untreated control cells but a significant downregulation of antiapoptotic gene Bcl2 indicating clear signs of apoptotic induction. The combined effect of SV-SOR showed the best apoptotic effect.

3.4. Effect of SV, SOR, and their combination on apoptosis and necrosis measured by flow-cytometry

Data presented in Fig. (4) showed a significant induction in early, late, and total percentage of apoptosis as well as the percentage of necrosis under the single or combined effect of SV + SOR. The combination showed the best apoptotic and necrotic effects.

3.5. Effect of SV, SOR, and their combination on cell cycle

Data presented in Table (3) and Fig. (5a-d) revealed a significant increase in the DNA content of the G0/G1 of cell cycle under the effect of SOR as compared to the untreated group and other treated groups indicating cell cycle arrest at the G0/G1. On the other side, SV and SV-SOR showed a significant increase in the DNA content of the S phase as compared to untreated cont. and SOR groups indicating cell cycle arrest at the S phase.

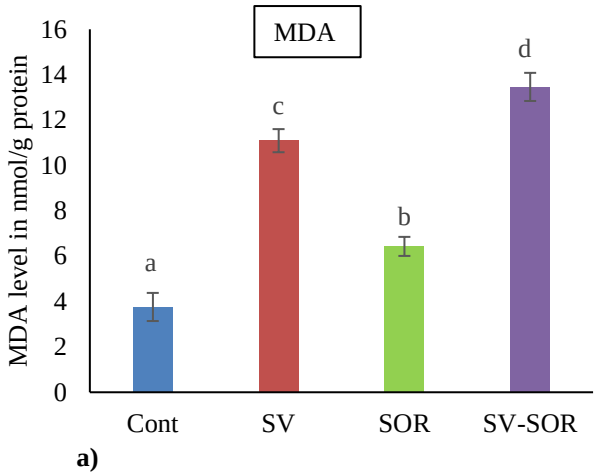
3.6. Effect of SV, SOR, and their combination on limiting enzymes of the glycolytic pathway

Data illustrated in Fig. (6a-c) showed a significant reduction in PK, ALDOB, and LDH activities to different degrees under the single or combined effect of SV + SOR as compared to untreated control. ALDOB showed the highest inhibitory effect. These data might indicate a significant reduction in the rate of glycolysis.

3.7. Effect of SV, SOR, and their combination on detoxifying enzyme ALDH1

As illustrated in Fig. (7) the present data revealed a significant elevation in ALDH1 detoxifying enzyme activity under the single or combined effect of SV + SOR as compared to untreated control indicating some type of resistance to their cytotoxic effect.

Fig. (2a-e): Effect of SV, SOR, and their combination on oxidative stress markers.



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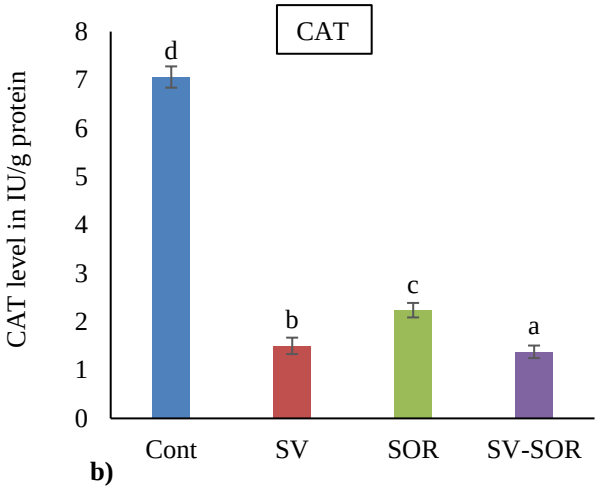

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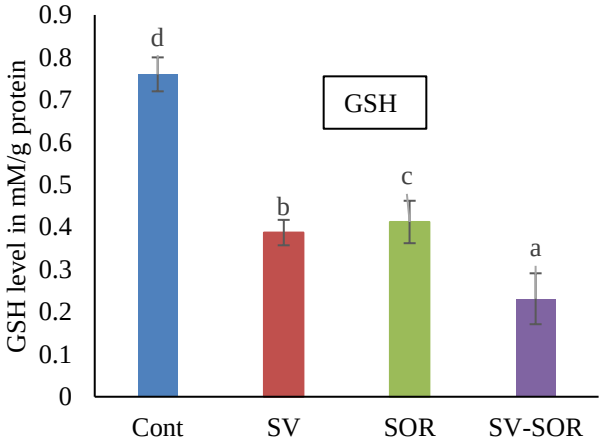

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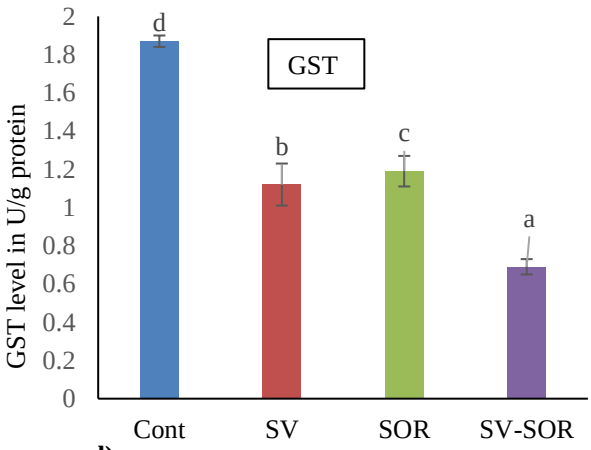

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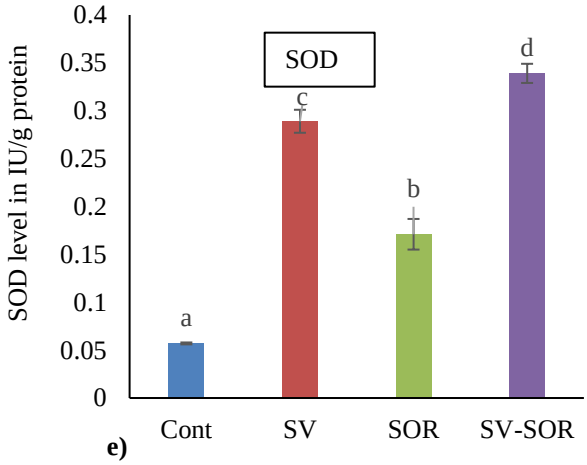


e)

Fig. (3a-d): Effect of SV, SOR, and their combination on gene expression of apoptotic markers.

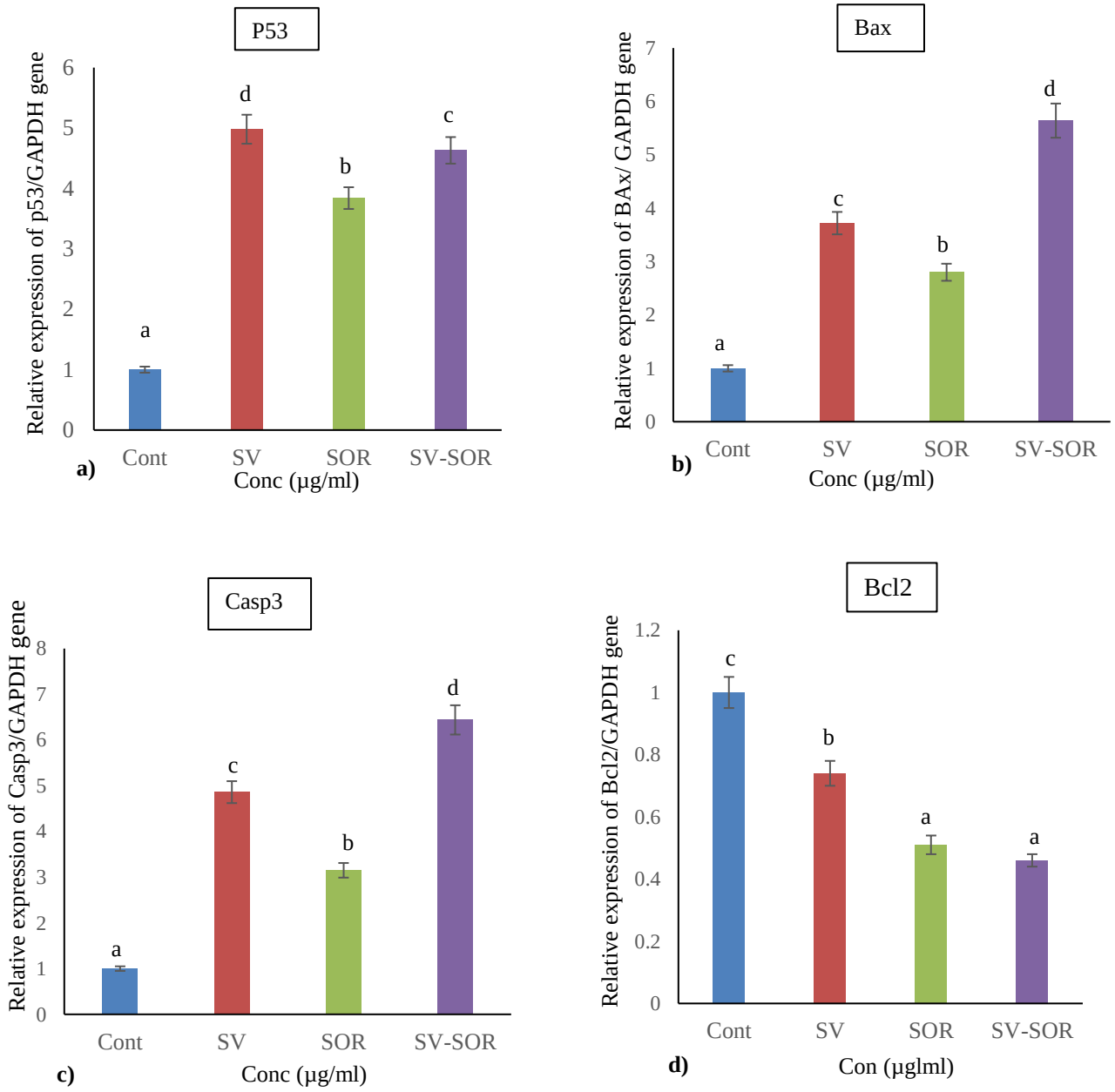


Fig. (4): Effect of SV, SOR, and their combination on apoptosis and necrosis measured by flow cytometry.

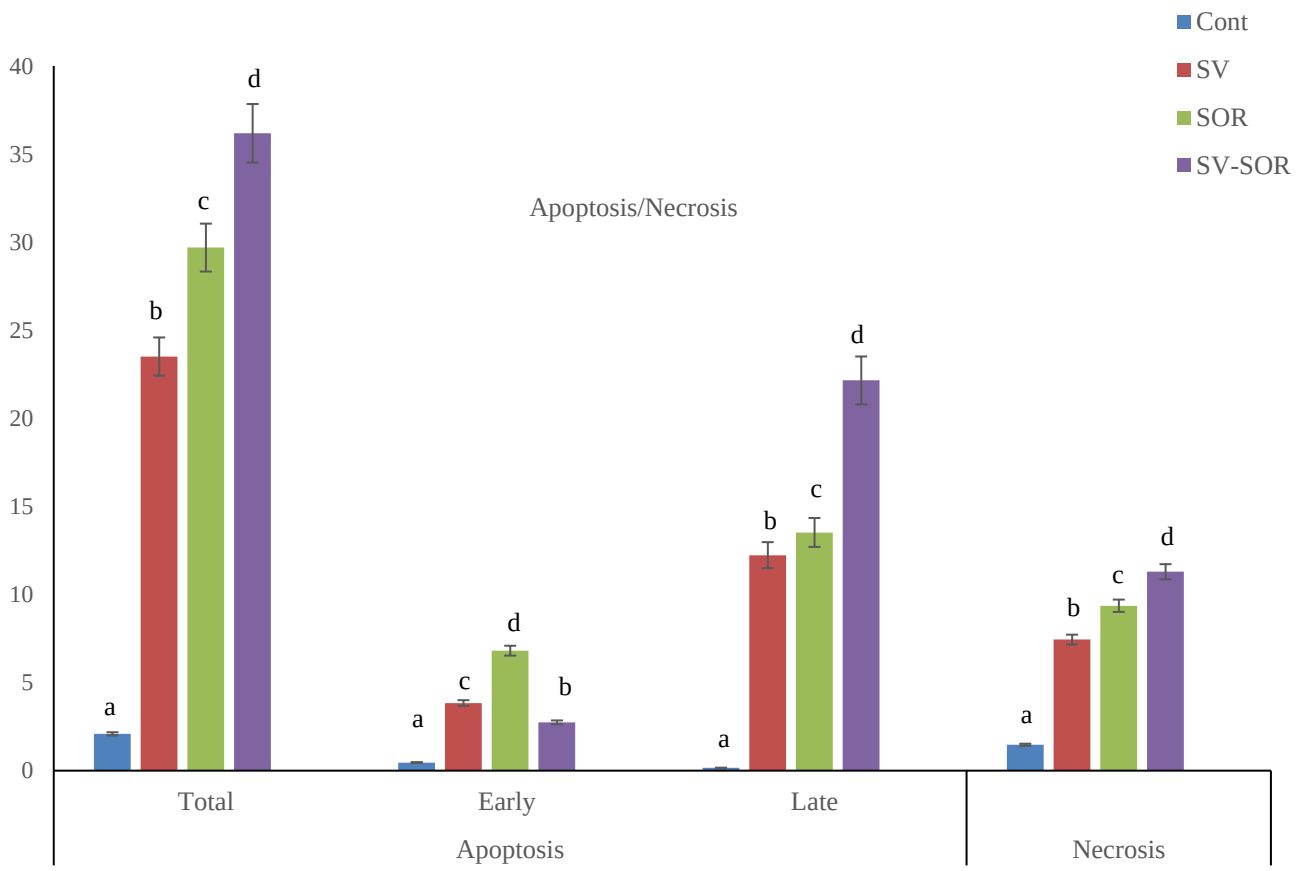
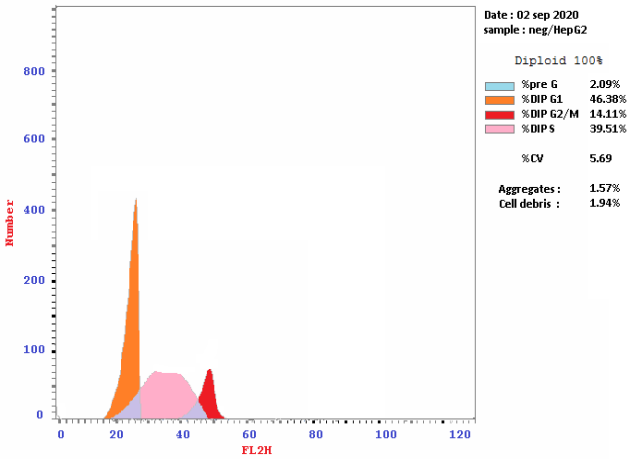


Table (3): Effect of SV, SOR, and their combination on cell cycle as measured by flow cytometry.

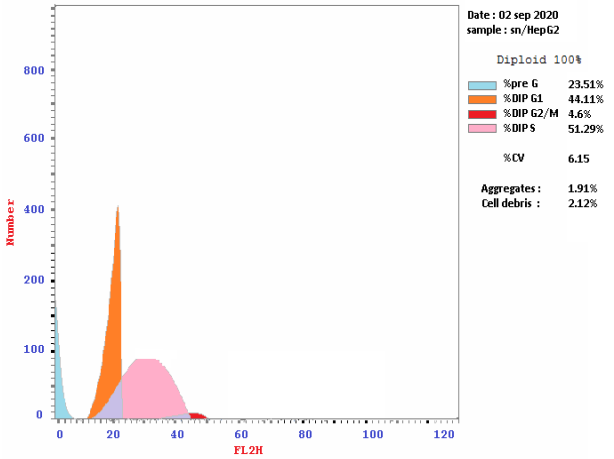
Sample	DNA content			
	%G0-G1	%S	%G2/M	%Pre-G1
Cont	46.38 ± 1.83[a]	39.5 ± 1.69[b]	14.11 ± 0.61[d]	2.09 ± 0.09[a]
SV	44.11 ± 1.74[a]	51.29 ± 2.20[c]	4.60 ± 0.20[a]	23.5± 1.10 [b]
SOR	53.16 ± 2.10[a]	34.26 ± 1.47[a]	12.58 ± 0.55[c]	29.69 ± 1.40 [c]
SV-SOR	37.09 ± 1.46 [a]	55.21 ± 2.37[d]	7.70 ± 0.33[b]	36.18 ± 1.70 [d]

Fig. (5a-d): Flow cytometric analysis of cell cycle under the effect of SV, SOR, and their combination

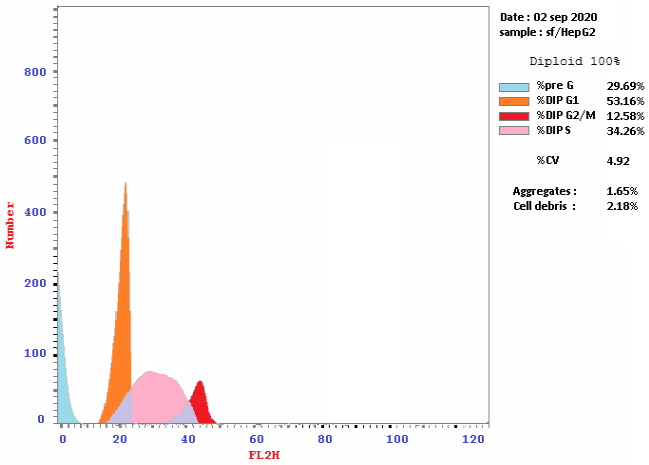
a) Control



b) SV



c) SOR



d) SV-SOR

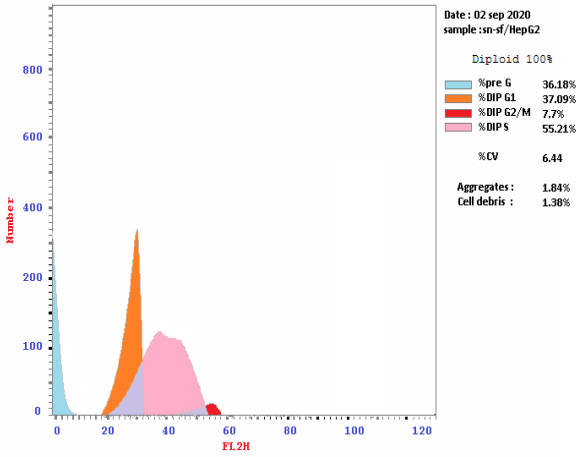
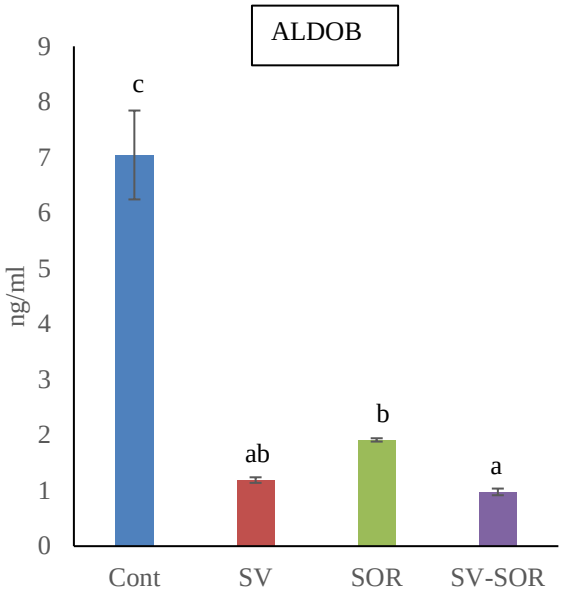
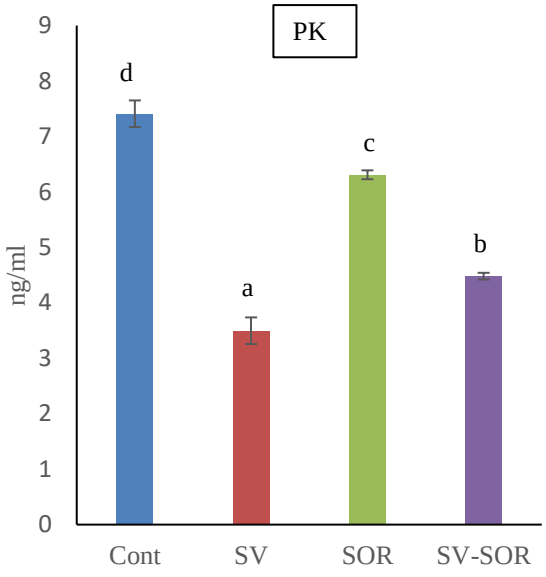


Fig. (6a-c): Effect of SV, SOR, and their combination on limiting enzymes of the glycolytic pathway.



a)



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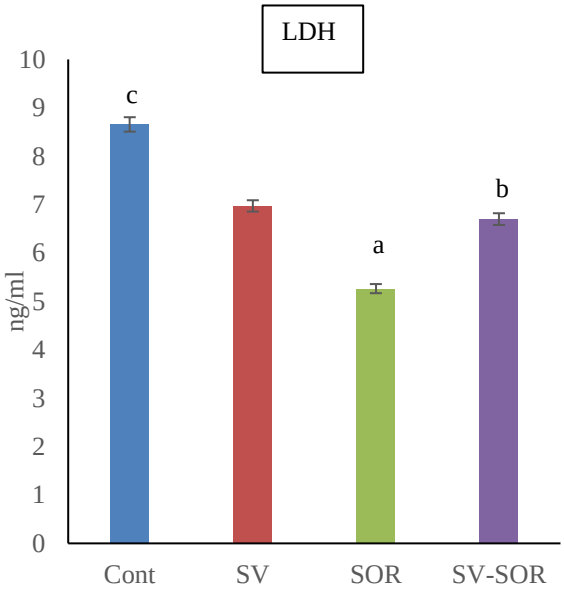
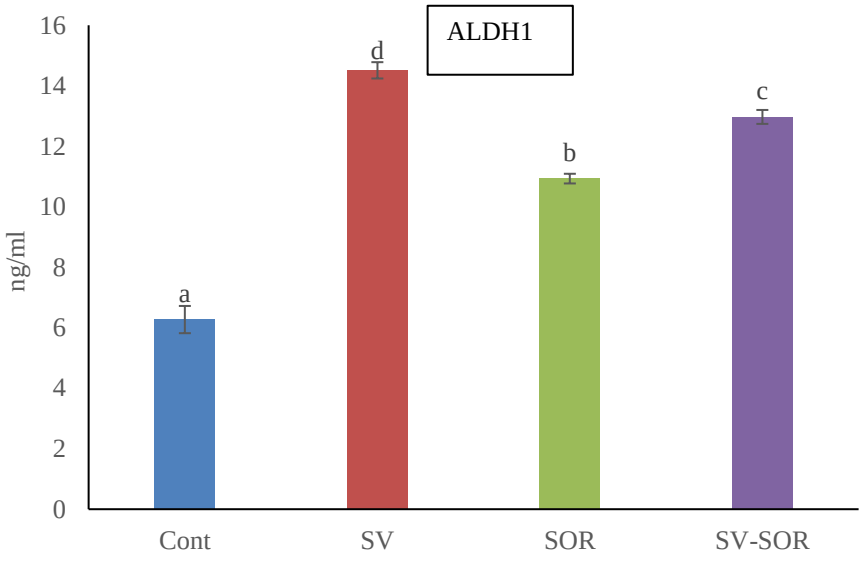


c)

Fig. (7): Effect of SV, SOR, and their combination on detoxifying enzyme ALDH1.



4. DISCUSSION

Sorafenib, the conventional chemotherapeutic drug for HCC is a multi-kinase inhibitor that interferes with intracellular and extracellular signaling pathways associated with tumor proliferation and angiogenesis **(Hajiev et al. 2021)**. It acts by inhibition of the Raf-MAPK pathway and the transcription/translation factors, such as NF-κB, STAT3, eIF2, and eIF4e, which eventually leads to the downregulation of anti-apoptotic Bcl-2 molecules **(Ricci et al. 2007, Rosato et al. 2007, Gillissen et al. 2017)**.

The harsh side effects as well as chemoresistance usually accompanying the use of synthetic chemotherapeutic drugs have increased the demand for new anticancer drugs derived from natural sources with high specificity and selectivity to cancer cells rather than normal cells.

The biodiversity of animals that use venoms and toxins as a defense system against their enemies makes them a source from which it is possible to create new therapeutics. **Aaghaz et al. (2019)** mentioned that many proteins and peptides from venoms have potential anticancer agents that can cure different forms of malignancies. Additionally, **Roy and Bharadvaja (2021)** reported that snake, scorpion, and bee venoms have potential cytotoxic effects on tumor cells. Currently, **Mansour et al. (2021)** have proved the combinational therapeutic effect of bee venom or its active component melittin with sorafenib against hepatocellular carcinoma cell line (HepG2). Moreover, **Mahfouz et al. (2021)** have also proved the combinational therapeutic effect of snake venom or its active component L-amino oxidase with sorafenib against HepG2.

The present study aimed to evaluate the in vitro individual cytotoxic effect of crude bee venom (BV), scorpion venom (SCV), and snake venom (SV) along with sorafenib on human hepatocellular carcinoma cell line (HepG2) as well as normal human retinal

pigmented epithelial cells (RBE1). Snake venom which showed the best antiproliferative activity against HepG2 has been combined with sorafenib in five nonconstant ratios and the combination index (CI) has been evaluated to expose their synergistic or antagonistic action.

The best combination which proves the most synergistic cytotoxic effect on HepG2 cells (CI <1), while having the most antagonistic effect on RPE1(CI >1) has been selected for downstream analysis of oxidative stress, apoptosis, cell cycle, and the limiting enzymes of the glycolytic pathway.

The present data demonstrated that among the three crude venoms, the crude snake venom showed the best sensitivity on HepG2 with IC50= 5.46 µg/ml versus 12.58 µg/ml for sorafenib, while having IC50= 7.94 µg/ml and 5.88 µg/ml, respectively against RPE1. These findings indicate the sensitivity of both liver cancer cells and normal cells to individual effects of either crude snake venom or sorafenib. The combination in the ratio IC_{25} SV+ C_{10} SOR appeared to be more sensitive and selective with 80.21% cell death on HepG2 (CI= 0.209) indicating a synergistic cytotoxic effect but with 10.13% cell death on normal RPE1 (CI=12.8) indicating an antagonistic effect. This last finding reflects the great safety of the selected combination of SV and SOR on normal cells. In this context, **Mahfouz et al. (2021)** came to the same conclusion by combining l-amino oxidase (the active ingredient of the crude snake venom) with sorafenib on HepG2 against the normal human liver cell line (THLE2).

Previous studies by **Zargan, et al. (2020)** showed the cytotoxic effect of Persian horned viper venom on the HepG2 cell line through induction of apoptosis and necrosis. **Lafnoune et al. (2021)** showed the anticancer effect on the HCC of Moroccan cobra *Naja haje* venom and its fraction obtained by gel filtration chromatography against the Huh7.5 cancer cell line. In addition, **Feofanov et al. (2004)** claimed that the cytotoxins from some snake species (N. haje, N. oxiana, and N. kaouthia) can penetrate living cancer cells as human lung adenocarcinoma A549, promyelocytic leukemia HL60 and markedly accumulate in lysosomes, which are the main targets of cytotoxins, and cause lysosomal leakage and plasma membrane injury.

In vivo, studies have also proved the potential of snake venom as an anticancer against different types of cancers. According to **Sun et al. (2003),** cobra venom apoptotically inhibited the growth of implanted hepatocellular carcinoma cells in mice. According to **Marcussi et al. (2007),** many toxins from snake venom have been formulated into drugs for the treatment of cancer.

The most important benefits of using the combination of a chemotherapeutic drug with venom is to reduce the required therapeutic dose of either; to minimize their side effects and also to decrease the cancer cell resistance. This hypothesis has been previously confirmed by the work of **Gajski et al. (2014)** in which BV has been combined with cisplatin for the treatment of human cervical and laryngeal carcinoma cells.

The present investigation revealed a significant anti-proliferative activity of single and/or combined SV with SOR on HepG2. Given the fact that most adverse side effects of SOR are attributed to its high and prolonged doses used in clinical applications, it is supposed that its co-administration at low doses with natural adjuvant, like SV used in the present study, might hypothetically inhibit cell proliferation and reduce its harsh side effects. Among the 5 tested combinations, only (IC_{25} SV + IC_{10} SOR) has fulfilled the following three criteria a) > 80% cell death, b) CI<1, and c) lower SOR dose, which have been selected for downstream analysis.

Fortunately, this combination showed very low toxicity against normal retinal pigment ephitilial-1cell line (RPE1). From the present findings, one can infer that a combination of SV with SOR has a synergistic anti-

proliferative effect (CI<1) even at a lower dose of SOR. This finding is supported by the recent work of **Mahfouz. et al. (2021)** on the combination of L-amino oxidase (the active ingredient of snake venom) with sorafenib, when applied to HepG2 cells with (CI<1). In this context, **Mansour et al. (2021)** have also confirmed the efficiency of combined melittin (an active ingredient of bee venom) when applied to HepG2 cells with (CI<1).

According to Liu et al. (2021), oxidative stress is an important factor in triggering apoptosis, which induces the action of a cascade of reactive oxygen detoxification systems. If the balance of pro-oxidant stress and anti-oxidant defenses becomes overwhelmed that could result in cell death. In the present study, crude SV, SOR, and their combination have been screened for their effect on oxidative stress markers including MDA, CAT, SOD, GSH, and GST. As compared to the individual effects, the combined treatment with SV + SOR seems to synergistically induce a significant increment in redox cycling and generation of ROS in terms of lipid peroxidation (MDA). As regards the antioxidants defense system, the present findings showed a significant increase in SOD but a significant decrease in CAT, GSH, and GST versus the untreated control indicating an overall accumulation of hydrogen peroxide.

SOD is known to catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. According to **Venugopal and Jaiswal (1996)**, measurement of SOD activity is an indirect method of detecting ROS, hence SOD activity reflects superoxide production in cells. The elevation encountered in the present study in SOD activity is an indicator of the high production of H_2O_2. This last finding might also indicate increased cellular resistance to chemotherapy. This remark is also confirmed by **Kumar and Clair (2021)** who claimed that increased SOD levels in prostate cancer cells in response to radiotherapy, is an indication of cellular resistance.

On the other hand, the significant reduction in CAT activity, which catalysis the conversion of H_2O_2 into water and oxygen, detected in the present study might lead to further accumulation of intracellular H_2O_2. **Bai and Cederbaum (2001)** demonstrated that the excessive production of hydrogen peroxide in mitochondria will damage lipids and proteins which can then cause cells to die of necrosis or apoptosis. The present findings run also in parallel with those reported by **Qin et al. (2019)** who showed a reduction of GSH in HepG2 cells treated with the phenolic composition of wild apricot and this seems to be associated with the induction of apoptosis. In addition, **Abdelglil et al. (2021)** claimed that the decrease in GSH in irradiated Cerastes cerastes snake venom-treated lung (A549) and prostate (PC3) cancer cells along with an increase in ROS values shifted the redox balance in favor of apoptosis.

Moreover, **Gao et al. (2012)** found that exposure to SW116 (colorectal cancer cells) to oridonin led to an increase of hydrogen peroxide and a decrease in GSH which affected the ROS level. **Siddiqui et al. (2013)** showed that rotenone depleted intracellular GSH, and catalase, and increased the level of MDA in HepG2 cells, which seems to be the same cause of cytotoxicity. Many studies have also demonstrated that hydrogen peroxide plays an important role in chemotherapy-induced apoptosis and senescence in cancer cells **(Engel and Evens 2006, Raj et al., 2011, and Shaw et al. 2011)**.

It is well known in the literature that SOR induces its cytotoxic effect by apoptosis secondary to the liberation of intracellular H_2O_2 as claimed by **Chiou et al. (2009) and Coriat et al. (2012)**. The present data has also confirmed the hypothesis that crude snake venom per se mediates its cytotoxic effect via accumulation of H_2O_2 by significantly increasing SOD and significantly reducing CAT, GSH, and GST versus both the untreated control and sorafenib. Hence the combination of both SOR and SV synergistically

stimulates H_2O_2 accumulation which is the main cause of apoptosis.

As regards GST, it is well known that glutathione S transferase is an antioxidant enzyme that catalysis a wide range of reactions especially detoxification of endogenous and exogenous harmful molecules by GSH conjugation. We detected here a significant inhibition in GST under the effect of SV and/or SOR as compared to untreated control. The greatest inhibition was encountered by a combination of SV IC_{25} + SOR IC_{10} indicating their synergistic inhibitory effect. This finding might also indicate the cytotoxic effect of SV/SOR on HepG2 cells.

The correlation between the cytotoxic effect of SV and/or SOR, excessive production of H_2O_2 via increased SOD and decreased CAT, and apoptosis is apparent in the present study, where an upregulation of proapoptotic genes (p53, Bax, caspase 3) along with downregulation of anti-apoptotic gene Bcl-2 has been encountered. In this context **Soto-Mercado et al. (2018)** claimed that the lipid-soluble zinc metal chelator TPEN induces apoptosis in murine mammary adenocarcinoma (TS/A) cells in a dose-dependent manner via inhibition of CAT, GSH, and GST activities; production of intracellular H_2O_2 as well as induced p53 and caspase-3.

Meanwhile, **Park et al. (2012)** reported that a combination of snake venom toxin (vipera lebetina turanica) with TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) upregulates the expression of pro-apoptotic proteins Bax and caspase-3, 8 and 9 but down-regulates the anti-apoptotic protein Bcl-2 in TRAIL-resistant HT-29, A549, and HepG2 cells. In addition, **Guo et al. (2015)** demonstrated an upregulation of p53 in HepG2 cells treated with l-amino oxidase isolated from snake venom. According to **Mahmoud et al. (2019)**, an apoptotic profile of HepG2 treated with Echispyramidum venom (Viperidae) showed an upregulation of apoptotic genes (P53, Bax, and Casp-3) and downregulation of anti-apoptotic gene (Bcl-2) in a significant way compared with untreated cell control. Moreover, **Mahfouz et al. (2021)** reported that a combination of SOR with L-amino oxidase, the active ingredient of snake venom, with CI<1 stimulates apoptosis in HepG2 as manifested by upregulation of pro-apoptotic genes (P53, Bax, and Casp-3) along with down-regulation of anti-apoptotic gene (Bcl-2).

The flow cytometric analyses of cell cycle, apoptosis, and necrosis illustrated in the present study have also confirmed the synergistic antiproliferative effect of SV and SOR on HepG2 cells and provide additional evidence of their cytotoxic effect. The present data recorded a delay and arrest of the cell cycle at the S phase after the single and combined application of SV and SOR on HepG2 cells. In this regard, **Chen et al. (2009)** mentioned that cytotoxin isolated from *Naja naja atra* snake venom affected HepG2 cell growth and increased the percentage of apoptotic cells being associated with cell cycle arrest at S-phase. In addition, **Chien et al. (2010)** demonstrated that Taiwan cobra cardiotoxin induces apoptosis and S-phase arrest in oral squamous cell carcinoma Ca9-22 cells.

Moreover, **Klein et al. (2011)** showed that crud snake venom of Jararhagin increased the expression of cell-cycle related genes TP53, CDKN1A, and CDKN2A as well as apoptosis-related genes Casp3, Casp5, Casp6 and Casp8 in melanoma cell (SK-Mel-288). On the other side, **Mahfouz et al. (2021)** recorded that SOR alone or in combination with LAAO, the bioactive compound in snake venom, induces cell cycle arrest at G2/M but a delay and arrest of the cell cycle at the G0/G1 phase under the effect of LAAO alone. These contradictory findings concerning the phase of the cell cycle during which cell death occurs seem to be correlated with the different mechanisms of action and/or percentage of cell population/phase at the start of the application of the drug. However, it implies the same final results of cell death.

Rapidly proliferating tumor cells consume glucose at a higher rate compared to normal cells, and part of their glucose carbon is converted into lactate, even in an oxygen-rich environment; a condition referred to as the 'Warburg effect' or 'aerobic glycolysis' as claimed by **Warburg (1930)**. The regulation of glycolysis and glycolytic components underscore the biological significance of tumor glycolysis. Thus, targeting glycolysis remains attractive for therapeutic intervention. Many enzymes of the glycolytic pathway also play significant roles in several non-glycolytic processes that enable the cancer cells to meet other cellular demands **Valvona et al. (2016)**. Hence, downregulating the glycolytic pathway through the control of some of the limiting enzyme activities and metabolic products could trigger antitumor effects in vitro and in vivo as claimed by **Chen et al (2007)**.

The specific metabolic activities of some of the limiting enzymes in the glycolytic pathway, especially those of aldolase, pyruvate kinase, and lactate dehydrogenase were estimated in the present study under the individual or combined effect of SV and SOR in HepG2 cells. A significant inhibition in activities of ALDOB, PK, and LDH was encountered after treatment for 48 h against untreated control. These findings infer that the rate of glycolysis is suppressed and ultimately inhibits cell proliferation of HepG2 cells.

Deregulated levels of LDHs have been previously reported in multiple tumors, including breast cancer **(Kurpińska, et al., 2019)**, nasopharyngeal cancer **(Su, et al., 2017)**, and bladder cancer **(Koukourakis, et al., 2016)**. Similar results have been demonstrated in a previous study, correlating the downregulation of LDH with induction of apoptosis, cell cycle arrest, and suppression of metastasis in HCC cells as cited by **Sheng, et al (2012)**. Moreover, **Nabi et al. (2021)** claimed that taurine suppresses the limiting enzymes (Aldolase and LDH) of the glycolytic pathway in hepatocellular carcinoma cell line (HepG2), which are indirectly correlated with the anticancer activity of taurine.

Aldolase is one of the limiting enzymes in the glycolytic pathway that catalyzes the reversible reaction of converting fructose1,6-biphosphate into dihydroxyacetone phosphate and glyceraldehydes 3-phosphate **(Chang et al., 2018)**. Aldolases are differentially expressed in human tissue, and aberrant expression has been observed in several human diseases and cancer types **(Chang et al., 2018)**.

PK catalyzes the final rate-limiting and irreversible step in glycolysis, which produces pyruvate and ATP. Pyruvate kinase M2 (PKM2), plays one of the most important roles in the aerobic glycolysis pathway, which mediates the conversion of phosphoenolpyruvate to pyruvate to release energy, and thus, it provides favorable conditions for the growth of cancer cells **(Tamada et al., 2012)**. Besides metabolic reprogramming, PKM2 acts as a protein kinase transcriptional coactivator of genes that influence cell proliferation, apoptosis, and migration **(Dong et al., 2016 and Zahra, et al. 2020).**

Lactate dehydrogenase (LDH) is one of the key metabolic enzymes that play an essential role in the conversion of pyruvate to lactate and vice versa making it an important player in cancer metabolism **(Valvona et al., 2016)**. It has been established that the downregulation of PKM2 reduces cancer growth and induces cell death **(Spoden et al., 2008)**. **Zhao and Song (2022)** showed that a serine protease from Trichosanthes kirilowii inhibits cell proliferation by blocking aerobic glycolysis in hepatocellular carcinoma cells via blocking PKM2-dependent glycolysis. In the present study, a significant reduction in PKM2 enzyme activity has been documented in HepG2 cells after treatment with SV, SOR, and their combination.

From the above results of the limiting enzymes of the glycolytic pathway, one can infer that treatment with SV, SOR, and their combination induced a significant

inhibition in the Warburg effect as compared with untreated HepG2.

Despite all the previous promising cytotoxic effects of SV, SOR, and their combination documented in the present study, there seem to be some signs of chemoresistance indicated by elevated levels of the ALDH1 detoxifying enzyme. This enzyme oxidizes intracellular aldehydes and has a pivotal role in the inactivation of chemotherapeutics.

According to **Nishikawa et al. (2013),** ALDH1 acts as a modulator for cell proliferation, stem cell differentiation, and resistance to chemotherapeutic agents. Also, overexpression of ALDH has been observed in different types of cancers as claimed by **Ma and Allan (2011)**. Moreover, it has been demonstrated that overexpression of ALDH is related to drug resistance and poor prognosis of many cancers including esophageal, breast, lung, stomach, and colon cancer **(Tanei, et al. 2009)**. On the other hand, ALDH inhibitors can be considered promising adjuvant agents in cancer treatment as they reduce the activity of ALDH leading to a decrease in the detoxification of chemotherapy drugs and improving clinical response to these drugs as reported by **Abdou, et al. (2017)**.

5. Conclusion

Our results suggest a novel synergistic, and anti-proliferative effect of SV (IC_{25}) with SOR (IC_{10}) on HepG2 cells. This combination could serve as a potential tool for the development of a novel therapeutic approach against HCC. As demonstrated in Fig. (8), the mechanism of action of this combination includes the generation of oxidative stress, induction of apoptosis, cell cycle arrest as well and induction of aerobic glycolysis (Warburg effect).

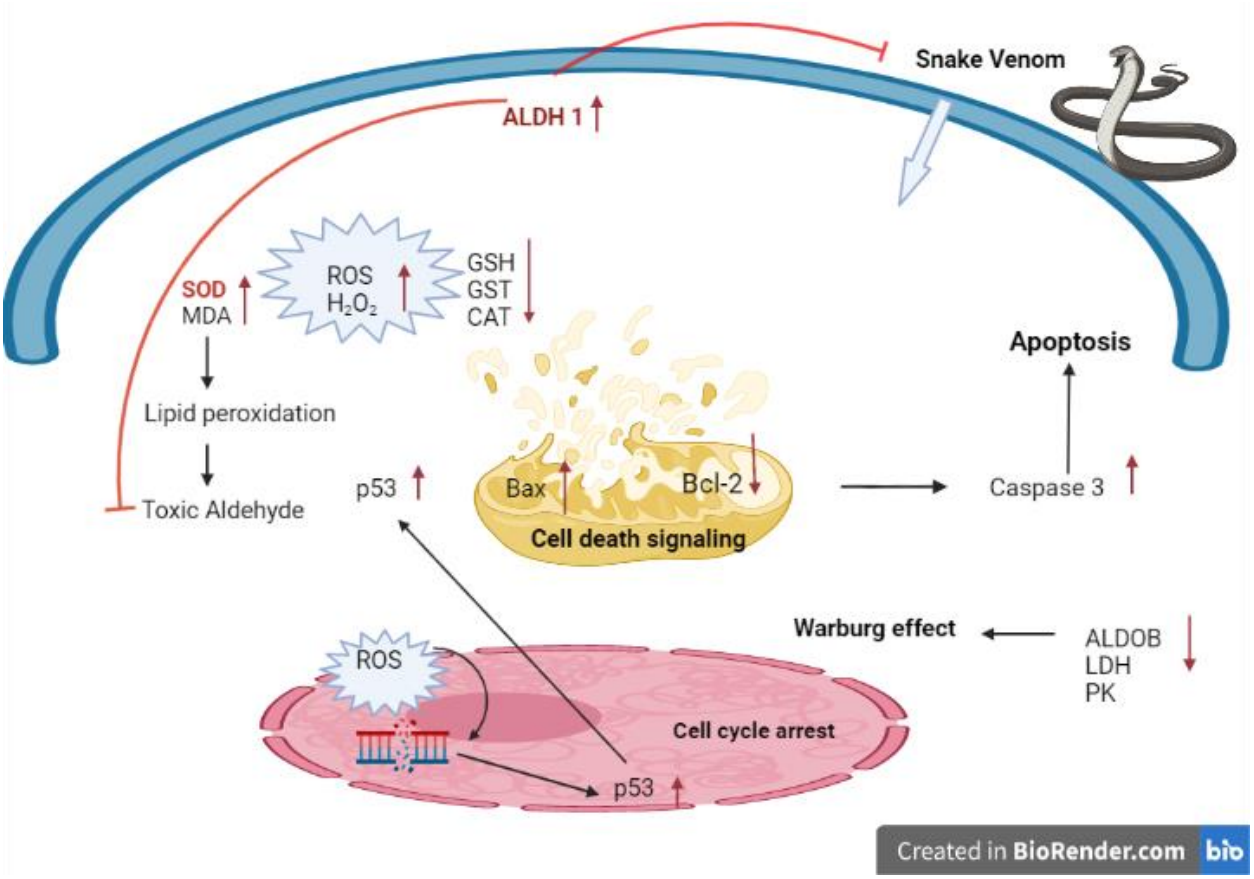
Conflict of interest:

None

Fund:

No funds, grants, or other support were received.

Fig. (8): Graphical abstract representing the mode of action of crude SV alone or combined with SOR.



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