# In-Vitro Cytotoxic and Antioxidant Activities of *Salvadora persica* in HepG2 Cells

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## ABSTRACT

Hepatic carcinoma (HCC) requires prompt clinical management to prevent morbidity and mortality. Current study was aimed to assess in-vitro cytotoxic, antioxidant and wound-healing effects of *Salvadora persica* (SP) various extracts, SP aqueous extract (SP-AQ), SP ethanolic extract (SP-E) and SP petroleum ether extract (SP-PE) on HCC cell line HepG2. In addition cytoprotective effect of SP extracts on normal cell line Vero was evaluated. Cytotoxic assays (MTT, LDH, Crystal violet and trypan blue), antioxidative enzymes assays (APOX, GSH, SOD and CAT) were performed on HepG2 and Vero cell line. Wound-healing assay on HepG2 cells was done. Results showed that SP different extracts exerted significant cytotoxic effect with antiproliferative activity and increased antioxidaive enzymes level in HepG2 cells. While SP extracts was observed harmless with non-significant cytotoxic effect in Vero cell line and also showed non-significant increase in antioxidative enzymes level in Vero cells. SP extracts at IC50 values did not heal wound in HepG2 cells. Based on these findings, plant SP could be an effective anticancer agent in hepatic carcinoma.

***Keywords*:** Hepatic carcinoma, cytotoxicity, antioxidant, In-vitro Technique

## 1. INTRODUCTION

HCC is fifth common encountered malignancy worldwide and ranks as third leading cause of malignancy associated deaths [1]. Recent international epidemiological report shows that primary hepatocellular carcinogenesis is occurred as 7$^{th}$ most frequent malignancy in males (4% of all malignancies) and in females represents the 13$^{th}$ most common malignancy (2.3% of all malignancies) [2]. HCC development is complex and different factors such as epigenetic and

genetic variants, inflammation and oxidative stress regulation are involved to carry out transformation of normal hepatocytes to cancer cells through multistep biological process [3]. Reactive oxygen species (ROS) can stimulate production of pro-inflammatory genes which causes induction of tissue injury due to production of hydrogen peroxide, superoxide and hydroxyl radicals. This increased oxidative stress and inflammatory lesion develop the pathogenesis of hepatic disease [4]. Cancer chemotherapy with conventional drugs causes several adverse effects. Whereas, plant derived products such as crude extracts have been showed better results with less side effects in prevention and treatment of cancer therapy [5]. Plant *Salvadora persica* (SP) belongs to family salvadoraceae. Plant SP is also known as toothbrush tree or miswak that is prepared from plant SP roots, twigs and stem. It was reported that decoctions and extracts of plant showed diversity of biological actions and have been used prophylactically and therapeutically to treat different ailments [6]. Current study was conducted to evaluate the hypothesis for plant SP that possesses the potential for cytotoxicity, anti-oxidant and wound-healing effects in HepG2 cells.

## 2. MATERIAL AND METHODS

### 2.1. Chemicals and Reagents

SP Miswak sticks (Raiwind road Lahore, Pakistan), Dulbecco's Modified Eagle's Medium-high glucose (DMEM-HG) (Caisson's Lab, Smithfield, USA), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) dye, Phosphate buffer saline (PBS), Trypan blue dye and crystal violet dye, sodium dodecyl sulphate (SDS) (Sigma-Aldrich, Louis, Missouri, USA).

### 2.2. Preparation of Plant Extracts

The selected plant was collected from the Raiwind road Lahore, Pakistan and its identification (voucher no. 3856) was done by Dr. Zaheer-ud-din khan, Distinguished Professor in Department of Botany, G.C University Lahore. The plant material was shade dried and powdered finally with a Chinese herbal grinder. Plant extracts were prepared using a cold maceration process. The ground plant material (100g) was soaked in 1 liter of distilled water to make SP-AQ extract, 70% ethanol to make SP-E extract and petroleum ether to make SP-PE for 72 hours at room temperature with occasional shaking. After three days, filtration of whole material was carried out by whatman # 1 in flask with tight cap and the filtrate was evaporated using rotary

evaporator under reduced pressure at 40ºC. The extract was then air-dried to obtain a solid mass. The crude extract color was light brown and it was also soluble in distilled water [7].

### 2.3. Culturing and Treatment of HepG2 and Vero Cell Lines

Hepatocellular carcinoma cell line, HepG2 and normal cell line, Vero were obtained from the cell culture laboratory which is established in The Department of Molecular biology and Biotechnology / Center for Research in Molecular Medicine (IMBB/CRIMM), The University of Lahore, Lahore. Both cell lines were cultured in DMEM-HG containing 10% FBS, 100 µg/ml streptomycin and 100 U penicillin at 95% humidity, 5% $CO_2$, 37 ºC [8].

### 2.4. Study Design

In cultured HepG2 and Vero cell lines, two major groups, control group and treated groups including SP-AQ extract treated group, SP-E extract treated group and SP-PE extract treated group were made. HepG2 and Vero cell lines on 2$^{nd}$ passage was cultured onto 96-well plate (NEST®, China) for cytotoxicity, antioxidant and LDH assays and on 6-well plate (NEST®, China) for wound healing assay.

### 2.5. Cytotoxicity Assays

2.5.1. MTT assay

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to assess 50% inhibitory concentration (IC50) and cytotoxicity of SP different extracts. SP extracts, SP-AQ, SP-E and SP-PE aliquots in concentrations of 10 µg/ml, 100 µg/ml, 200 µg/ml, 500 µg/ml and 1000 µg/ml were applied on HepG2 cell line in wells of SP extracts treated groups and incubated for 24 hours and then removed. Cultured HepG2 cells monolayer was washed with PBS for 3 times. Later on, 25 µl of MTT solution was added in wells of control group and treated groups and incubated for 2 hours. Then 10% sodium dodecyl sulphate (SDS) was added in wells to solubilize formazan. After 1 hour absorbance of solution in 96-well plate was taken at 570 nm by UV-spectrophotometer (Bio-Rad, USA). Same procedure was done for Vero cells to determine cytotoxicity of SP different extracts at IC50 values [9].

2.5.2. Lactate dehydrogenase (LDH) assay

LDH enzyme release activity was determined by taking harvested medium from control group and SP extracts treated group after 24 hour by using LDH assay kit (AMP Diagnostics, Austria). The procedure employed was in according with manufacturer's instructions. Precisely, in 96 well plate, 5 µl medium was taken from control group and SP extracts (SP-AQ, SP-E and SP-PE) treated groups that was mixed with 100 µl of working reagent and incubated for 5 minutes. Absorbance was measured by spectrophotometer at 340 nm.

2.5.3. Crystal violet assay

SP various extracts 100 µl at IC50 values were administered to HepG2 and Vero cells in treated groups and incubation was done for 24 hours. Cells monolayer was washed with PBS for 3 times. Further 0.1% of crystal violet mixed with 2% ethanol was added in all group wells to cover cell surface and it was left to incubate for 15 minutes. Then crystal violet dye was discarded carefully so that cells may not disappear from wells. Where adhered viable (live) cells were stained with crystal violet dye and non-viable (dead) cells lose their adherence during cell death resulting in reduced crystal violet dye amount in cultured cell population. After that 100 µl of 1% SDS was applied in each well to solubilize crystal violet stain and left for 15 minutes. Then absorbance reading of mixture of wells in plates was taken at 590 nm by microtiter plate [10].

2.5.4. Trypan blue assay

HepG2 cell line and Vero cell line was treated with 100 µl of SP all extracts at IC50 values and incubated for 24 hours. After that medium in all wells was removed following cells washing by PBS and washing was carried out for 3 times. Then 0.4% trypan blue dye at 100 µl was solubilized in PBS and 1:1 ratio was made. After that trypan blue dye, dissolved in PBS was administered to control group and SP extracts treated groups of HepG2 cell line and Vero cell line in wells for 10 minutes. Trypan blue dye was taken by compromised cell membrane of HepG2 cells and Vero cells during death that were stained blue and dead cells were counted. While viable cells excluded trypan blue dye. Then washing with PBS was done for 3 times. Cells stained with trypan blue dye were appeared blue and considered non-viable (dead) cells that were counted by observing under a microscope [10]. Following formula was applied to determine percentage of dead cells.

Percentage (%) of dead cells = Dead no. of cells in treated groups – dead no. of cells in control group x 100

**2.6. Antioxidant assays**

Antioxidant assays were performed as described by Maqbool et al [11]. For measurement of antioxidative enzymes level in harvested medium of HepG2 cell line and Vero cell line, two groups were made. One was pre-treatment group and second was post-treatment group. Harvested medium in pre-treatment group was collected at 0 hour from wells after application of SP different extracts, SP-AQ extract, SP-E extract and SP-PE extract. While in post-treatment group incubation with SP all extracts was done for 24 hours. Later on, harvested medium taken from both groups was mixed with reaction mixture and then processed. In pre-treatment group and post-treatment group, control group did not receive SP various extracts treatment.

2.6.1. Ascorbate peroxidase (APOX) assay

Reaction mixture was prepared by mixing 2.5 mM ascorbic acid, 25 mM $KH_2PO_4$ buffer (pH 7.0) and 75 mM $H_2O_2$. After that 100 µl of medium from pre-treatment group and post-treatment group was added in 200 µl of reaction mixture. Then it was mixed thoroughly and it was kept in light for 3 minutes. Measurement of optical density at wavelength 290 nm by spectrophotometer was done.

2.6.2. Glutathione reductase (GSH) assay

The 200 µl of reaction mixture was synthesized by mixing 40 mM EDTA, 20 mM $KH_2PO_4$ buffer (pH 7.5) and 10 mM oxidized glutathione then it was mixed with 100 µl of medium from pre-treatment and post-treatment groups. In the last step 20 mM NADPH was added and absorbance was taken at 340 nm by spectrophotometer.

2.6.3. Superoxide dismutase (SOD) assay

Reaction mixture was prepared by mixing 0.1mM EDTA, 100 mM KH2PO4 buffer (pH 7.8), 2.25 mM nitro blue tetrazolium chloride (NBT), 60 µM riboflavin, and 13 mM methionine. Then 200 µl of reaction mixture was mixed with 100 µl of medium, collected from pre-treatment and post-treatment groups. After 10 minutes in light exposure, optical density was determined at 560 nm against blank by spectrophotometer.

2.6.4. Catalase (CAT) assay

Reaction mixture was prepared by adding 31.25 mM $H_2O_2$ and 12.5 mM $KH_2PO_4$ (pH 7.0) then 200 µl of reaction mixture was mixed with 100 µl medium of pre-treatment group and post-treatment group. It was kept in light for 45 to 60 seconds. After that optical density was determined at 240 nm wavelength spectrophotometrically.

### 2.7. Wound-healing assay or scratch assay

HepG2 cells were plated in 6 well plate. Wound-healing assay was carried out by using sterile pipette tip to make straight scratch on monolayer of cells that stimulated the wound formation. Plant extracts (SP-AQ, SP-E and SP-PE extracts) were applied to the media in wells and incubated for 24 hours. During incubation, cells migration and cells accumulation were observed at an interval of 0 hour, 10 hours and 24 hours by taking images under an inverted microscope equipped with camera (Floid Cell Imaging Station, Life Technologies) [12].

### 2.8. Statistical analysis

All data of cytotoxicity and antioxidant assays are expressed in mean ± SEM with n = 3 experiments. Data of cytotoxicity assays was subjected to One-way ANOVA with Bonferroni's test with multiple comparisons. Data of antioxidant assays was analyzed by Two-way ANOVA with Bonferronis's test with multiple comparison. A *p*-value of 0.05 was considered significant.

## 3. RESULTS

### 3.1. Cytotoxicity assays

3.1.1. MTT assay

In MTT assay SP various extracts significantly decreased cellular proliferation in HepG2 cells in dose dependent manner and IC50 values of SP extracts were calculated including SP-AQ (SP aqueous extract) 567.9 µg/ml, SP-E (SP ethanolic extract) 521.2 µg/ml and SP-PE (SP petroleum ether extract) 488.8 µg/ml (Figure 1, a1-a3). SP all extracts showed non-significant number of cell death in Vero cell line (Figure 1b). In cytotoxicity assays SP-PE extract showed pronounced effect than SP-E and SP-AQ extracts.

3.1.2. LDH Assay

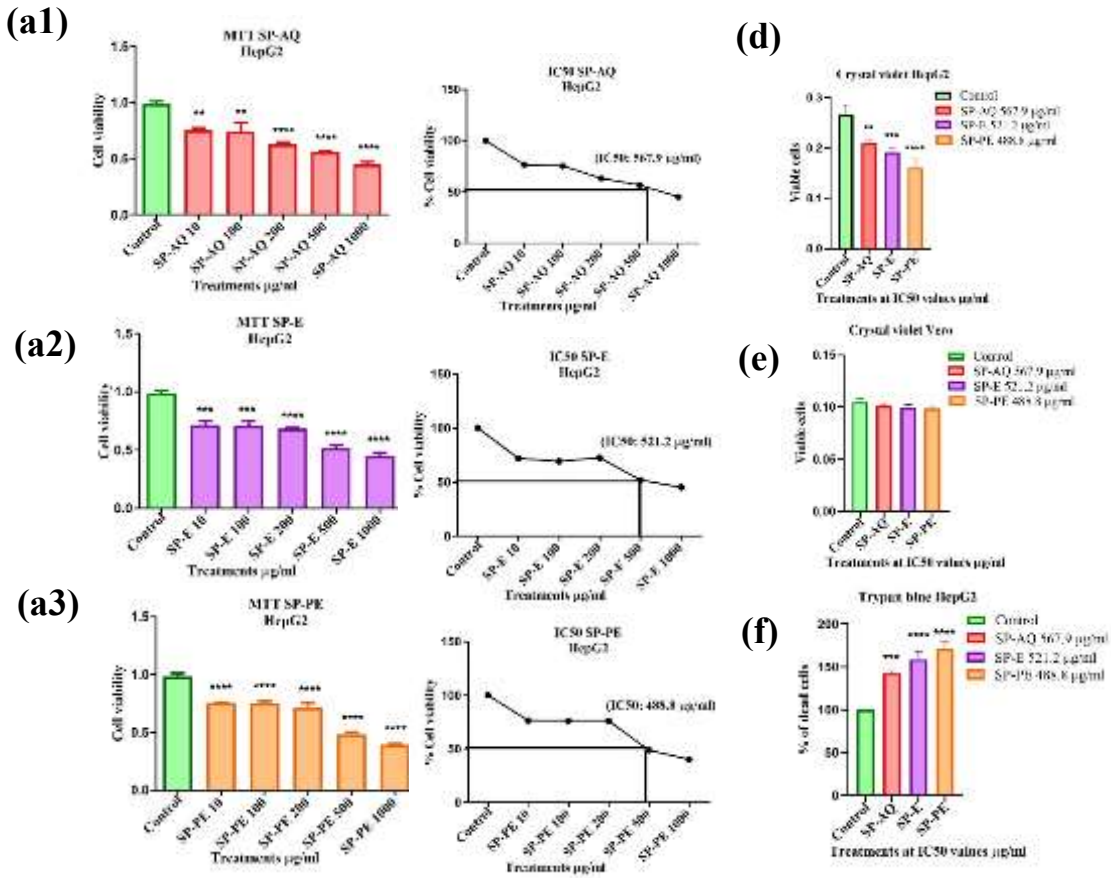
SP different extracts, SP-AQ, SP-E and SP-PE treated HepG2 cells were observed with significant increased level of enzyme lactate dehydrogenase than control group. SP-PE showed higher level of LDH release than remaining SP-E extract and SP-AQ extract (Figure 1c).

3.1.3. Crystal Violet Assay

SP various extracts SP-AQ, SP-E and SP-PE showed less number of viable cells with significant number of non-viable cells in treated HepG2 cells (Figure 1d). While non-significant number of non-viable cells were observed in Vero cells (Figure 1e).

3.1.4. Trypan blue assay

SP different extracts showed increased number of blue stained dead HepG2 cells with inconsiderable non-significant activity in Vero cell line (Figure 1f, g ).



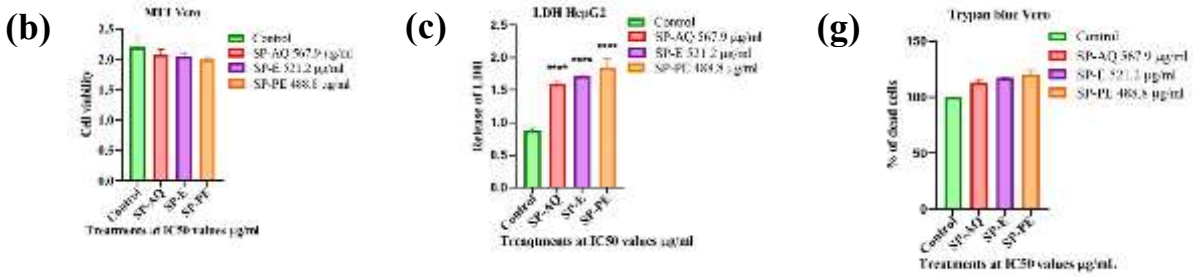


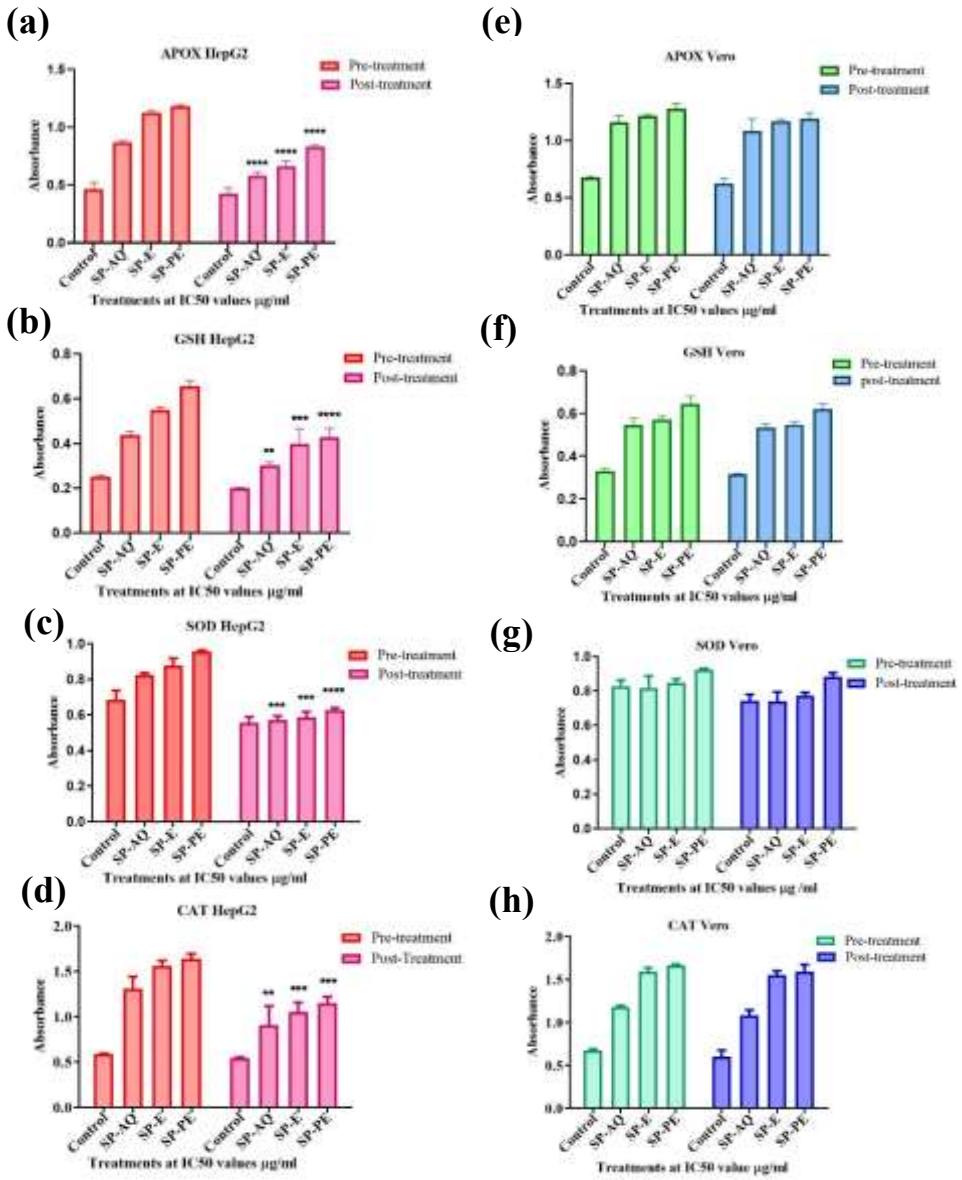

**Figure 1.** In-vitro cytotoxicity assays of HepG2 and Vero cells treated by *Salvadora persica* (SP) extracts: **(a1-a3)** Cell viability and 50% inhibitory concentration (IC50) of SP extracts, SP aqueous extract (SP-AQ), SP ethanolic extract (SP-E) and SP-petroleum ether extract (SP-PE) on HepG2 cell line and **(b)** Vero cell line were determined by MTT assay **(c)** LDH assay at IC50 values of SP extracts was done in HepG2 cells to observe LDH enzyme release level **(d,e)** Crystal violet assay at $IC_{50}$ values of SP extracts was performed in HepG2 cell line and Vero cell line to evaluate number of viable cells and **(f,g)** trypan blue assay at SP extracts $IC_{50}$ values was done to determine % of dead cells in SP extracts treated groups from control group in HepG2 cell line and Vero cell line. In cytotoxicity assays decreased number of viable cells were observed in treated HepG2 cells while treated Vero cells remained safe when compared with control group. Data represented are mean ± SEM with n=3. One way ANOVA, Bonferroni's test with multiple comparison was applied on data. (**$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

### 3.2. Antioxidant Assays

SP extracts, SP-AQ, SP-E and SP-PE showed significantly increased level of antioxidative enzymes, APOX, GSH, SOD and CAT in pre-treatment group and post-treatment groups as compared to control group of HepG2 cell line. Although more increased antioxidative enzymes (APOX, GSH, SOD and CAT) level was observed in pre-treatment group of HepG2 cell line (Figure 2a,b,c,d) than post-treatment group. Among SP extracts, SP-PE extract was observed most efficient in antioxidant potential than SP-AQ extract and SP-E extract. While reduced antioxidant activity was showed by SP-AQ extract. The non-significant increased antioxidant potential of SP all extracts was found in treated groups of Vero cell line (Figure 2e,f,g,h).

**Figure 2**. Antioxidant enzymes assay: **(a,b,c,d)** SP different extracts, SP aqueous extract (SP-AQ), SP ethanolic extract (SP-E) and SP-petroleum ether extract (SP-PE) at IC50 values

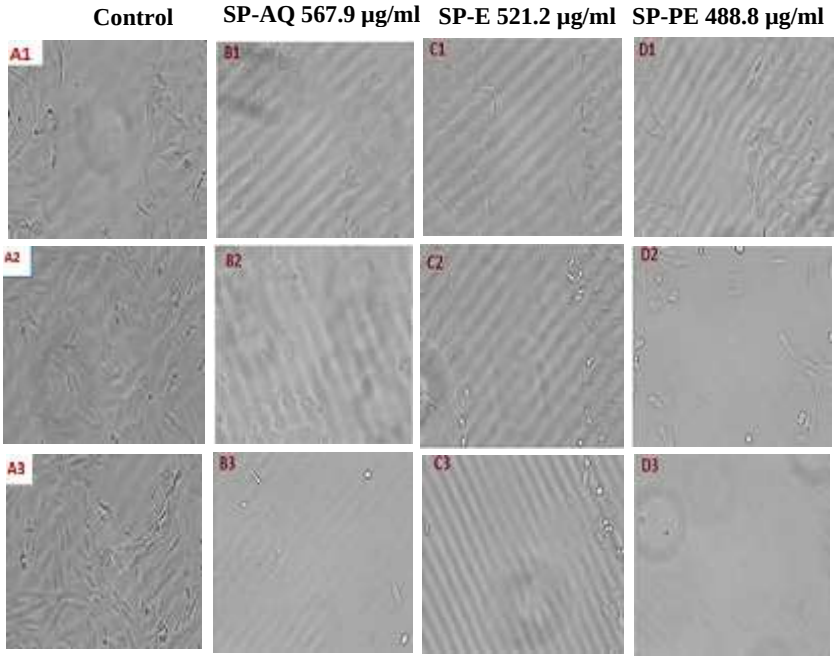
increased antioxidative enzymes level of ascorbate peroxide (APOX) and glutathione reductase (GSH), superoxide dismutase (SOD) and catalase (CAT) in HepG2 cell line by taking harvested medium after incubation with SP extracts for 0 h in pre-treatment group and 24 h in post-treatment group. **(e,f,g,h)** In Vero cell line non-significant increased level of antioxidant enzymes were observed. Values are expressed as mean ± SEM and Two-way ANOVA with Bonferronis's test with multiple comparisons was applied. (**$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).




### 3.3 Wound-healing Assay (Scratch Assay)

SP different extracts SP-AQ, SP-E and SP-PE at IC50 values did not heal wound in SP extracts treated HepG2 cells, observed at 12 hours and also at 24 hours from 0 hour of treatment. Whereas wound-healing was observed in control group of HepG2 cells (Figure 3).

**Figure 3.** Wound-healing assay: *Salvadora persica* (SP) different extracts, SP aqueous extract (SP-AQ), SP ethanolic extract (SP-E) and SP-petroleum ether extract (SP-PE) at IC50 values did not show healing of wound in treated HepG2 cells groups as compared to control group.




## 4. DISCUSSION

Natural plants in the total extracts form or their isolated purified functional compounds demonstrate an integral role in current prevention and treatment of various cancers [13]. Natural compounds inhibit various mechanisms of cancer development and stimulate different mechanisms such as anti-tumor, anti-proliferative and antioxidant that provide effective therapeutic strategies for treating cancer. Many natural compounds exert selective cytotoxic effect on cancer cells and do not affect non-cancerous or normal cells [14]. The aim of present study was to determine in-vitro possible mechanisms of anticancer activity of *Salvadora persica* (SP) roots (miswak) different extracts, SP aqueous extract (SP-AQ), SP ethanolic extract (SP-E) and SP petroleum ether extract (SP-PE) on HCC cell line HepG2 by focusing on determination of cytotoxic assays (MTT, crystal violet, trypan blue and LDH), antioxidative enzymes assays

(APOX, GSH, SOD and CAT) and wound-healing effect. MTT assay is calorimetric assay for quick assessment of cytotoxicity and cellular proliferation by measuring cell metabolism. The living cells with active metabolism reduce MTT to purple color formazan crystals that correlates with cells number [15]. In MTT assay various SP extracts showed significant cytotoxicity with IC50 values, SP-AQ = 567.9 µg/ml, SP-E = 521.2 µg/ml and SP-PE = 488.8 µg/ml respectively against HepG2 cell line (Figure 1: a1,a2,a3). These IC50 values are acceptable as another study has reported cytotoxic activity of plant *Moringa oleifera* seeds essential oil with IC50 value 751.9 µg/ml against HepG2 cell line [16]. LDH assay is metabolic cellular proliferation assays to evaluate cytotoxic activity of drugs [17]. Lactic dehydrogenase (LDH) is a glycolytic enzyme that is released from necrotic tissue [18]. Some previous studies shows that more LDH is released from anticancer plant treated groups compared to control group [19]. SP extracts, SP-AQ, SP-E and SP-PE in treated HepG2 cells increased release of LDH level (Figure 1c). In other cell viability assays, SP extracts (SP-AQ, SP-E and SP-PE) showed less number of viable HepG2 cells in crystal violet assay (Figure 1d) and increased number of HepG2 dead cells were observed in trypan blue assay (Figure 1f). In the current study, SP-PE extract was found most effective cytotoxic on HepG2 cells. These results are similar as previously described [20]. In addition, SP-AQ, SP-E and SP-PE extracts revealed least cytotoxic effect on normal cell line Vero using MTT, crystal violet and trypan blue assays (Figure 1b,e,g). Which means that SP extracts are safer in normal cells which may be a good point for a cytotoxic drug in cancer treatment [21]. Reactive oxygen species (ROS) induce oxidative stress that is responsible for uncontrolled division of cells and mutations [22]. Antioxidant enzymes including APOX, GSH, SOD and CAT show radical scavenging activity and decrease ROS [23]. In the present study, antioxidant potential of SP-AQ, SP-E and SP-PE extracts was measured in medium and it was found that antioxidant activity increased the levels of detoxifying enzymes (APOX, GSH, SOD and CAT) and reduced oxidative stress in HepG2 cells (Figure 2a,b,c,d). Antioxidant enzymes activity in pre-treatment group and post-treatment groups was high as compared to control group but in pre-treatment group this antioxidant activity was more high because SP extracts concentration in pre-treatment group medium at 0 hour was not consumed to affect HepG2 cells whereas in post-treatment group HepG2 cells were incubated for 24 hours with SP different extracts, whose maximum concentration during 24 hours incubation was consumed by HepG2 cells to cause cytotoxic effect and less amount of SP extracts was available in medium to show

antioxidant potential. These antioxidant enzymes assay methodology and results are in accordance with another plant study results in which *Nigella sativa* extract showed higher antioxidative enzymes (APOX. GSH, SOD and CAT) level in pre-treatment group than post-treatment group [11]. While in Vero cell line SP various extracts showed non-significant increase in antioxidative enzymes level. In wound-healing assay SP-AQ, SP-E and SP-PE extracts at IC50 concentrations in treated HepG2 cells did not show wound-healing effect in comparison to control group in which wound healing was seen (Figure 3). This may be linked with significant cytotoxic effect of SP extracts that is responsible for inhibition of wound-healing in treated HepG2 cells. Our wound healing assay results are in accordance with another study in which cytotoxic plant *Cratoxylum formosum* leaf extract inhibited wound healing in HepG2 cell line [24]. In current study SP-PE extracts showed more cytotoxic and antioxidant effects which might be related that SP-PE extract is rich in some phytocompounds than remaining SP extracts.

## 5. CONCLUSION

Current study showed that SP root different extracts (SP-AQ, SP-E and SP-PE) possess in vitro cytotoxic, antiproliferative and antioxidant activities and further study should be done to explore phytochemicals and mechanisms responsible for anticancer activity in hepatocellular carcinoma.

**Conflict of Interest:** The authors declare that there is no conflict of interest regarding this study.

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