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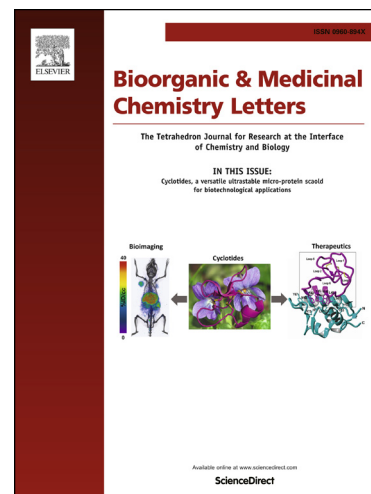
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Synthetic Strigolactone Analogues Reveal Anti-Cancer Activities on Hepatocellular Carcinoma Cells

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Abstract:

Hepatocellular carcinoma (HCC) remains one of the leading causes of death worldwide. The complex etiology is attributed to many factors like heredity, cirrhosis, hepatitis infections or the dysregulation of the different molecular pathways. Nevertheless, the current treatment regimens have either severe side effects or tumors gradually acquire resistance upon prolonged use. Thus, developing a new selective treatment for HCC is the need of the hour. Many anticancer agents derived from plants have been evaluated for their cytotoxicity towards many human cancer cell lines. Strigolactones (SLs)-a newly discovered class of phytohormones, play a crucial role in the development of plant-root and shoot. Recently, many synthetic analogues of SLs have demonstrated pro-apoptotic effects on different cancer cell lines like prostate, breast, colon and lung. In this study, we tested synthetic SLs analogues on HCC cell line-HepG2 and evaluated their capability to induce cell proliferation inhibition and apoptosis. Primary WST-1 assays, followed by annexin-V/7AAD staining, demonstrated the anti-proliferative effects. The SLs analogues **TIT3** and **TIT7** were found to significantly reduce HepG2 cell viability in a dose- and time-dependent manner and induce apoptosis. Interestingly, though **TIT3** and **TIT7** strongly affected cancer cell proliferation, both compounds showed moderate anti-proliferative effect on normal cells. Further, migration of cancer cells was suppressed upon treatment with **TIT3** and **TIT7** in a wound healing assay. In summary, these findings suggest that two SLs analogues **TIT3** and **TIT7** exert selective inhibitory effects on cancer cells most likely through targeting microtubules. SLs analogues could be used in future as potential anti-cancer candidates in chemotherapy.

Keywords: Strigolactones, Liver cancer, Apoptosis, Cell proliferation, Hepatocellular carcinoma.

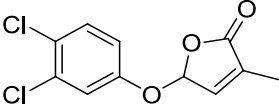
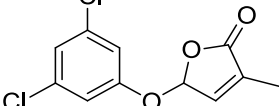
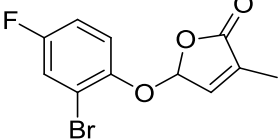
Hepatocellular carcinoma (HCC) is the predominant form of primary malignant liver tumor accounting to 80-90% of all liver cancers.¹⁻³ Yearly, one million cases of HCC are diagnosed worldwide, and more than 690,000 of them consequently die.⁴ HCC is the fifth most common type of cancer in men and eighth in women⁵ and is considered as the third leading cause of cancer-related deaths globally.⁶ Several factors could be involved in the development of hepatic tumors including chronic hepatitis B and C viral infections, diabetes mellitus and obesity.^{3,7-9} Many studies have illustrated that patient survival rates can be significantly increased if tumors are detected at early stages,^{10,11} and the subsequent treatments such as liver transplantation and resection are timely done.¹² Furthermore, chemotherapy is more frequently encountered with resistance, and nearly no effective cure is found in advanced HCC stages.¹¹

Lack of understanding the molecular mechanisms of hepatic tumors limits the development of practical therapeutic approaches for it.¹³⁻¹⁵ The significant drawbacks of the current treatment regimens like severe side effects, off-target toxicities and gradually acquired resistance by tumors upon prolonged use remains a challenge. Therefore, there is a pressing demand of developing new drugs and investigate their anti-proliferative effects on HCC to treat patients.

In the recent few years, many anticancer agents derived from plants have been evaluated for their potential to inhibit the proliferation of many human cancer cell lines.¹⁶⁻²¹ Strigolactones (SLs) are a novel class of plant hormones, responsible for root and shoot development. Many synthetic analogues of SLs have demonstrated pro-apoptotic effects on a panel of cancer cell lines including osteosarcoma, leukaemia, lung, colon, prostate and breast.²²⁻²⁴ These synthetic SLs analogues were found to induce a G2/M arrest and apoptosis in cancer cells.^{22,24} Interestingly, the tested SLs analogues showed only a miniscule inhibition against

normal cells (BJ fibroblasts).²⁴ SLs analogues have been shown to inhibit cell proliferation and induce apoptosis by targeting several signalling pathways. In this context, treatment of colon, prostate²² and breast²⁴ cancer cell lines with synthetic SLs analogues caused the activation of p38 and JNK1/2 mitogen-activated protein kinases (MAPKs) leading to the inhibition of ERK1/2 and PI3K/ AKT; the consequence was cell cycle arrest at G2 phase.^{22,24} A recent study has reported that SLs analogues were able to destabilize genomic DNA in osteosarcoma cells inducing double-strand breaks (DSBs) and thereafter, activated DNA damage response. This effect was associated with inhibition of DNA repair resulting in cell death.²⁵ Interestingly, neither DSBs nor cell death has been reported in healthy BJ fibroblast cells suggesting the critical clinical relevance of SLs analogues in cancer therapy.

In the present study, we evaluated the anti-proliferative and pro-apoptotic activities of designed SLs analogues (Supporting Information) on HCC. Our results have shown that among the tested SLs analogues, two compounds, **TIT3** and **TIT7** were able to induce cell proliferation inhibition and apoptosis in HCC cells through dose- and time-dependent mechanisms with minor inhibitory effects on healthy cells. We also found that the SLs analogues **TIT3** and **TIT7** can inhibit the migration of hepatic tumor cells.

SL Analogue	Molecular Formula; Weight	Structure
TSK13	C ₁₁ H ₈ Cl ₂ O ₃ ; 259.09	
TSK14	C ₁₁ H ₈ Cl ₂ O ₃ ; 259.09	
TSK15	C ₁₁ H ₈ BrFO ₃ ; 287.08	

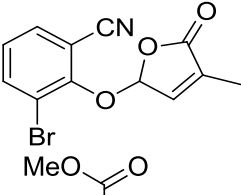
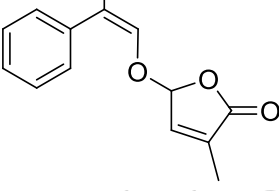
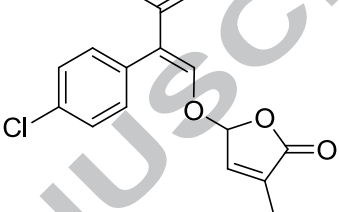
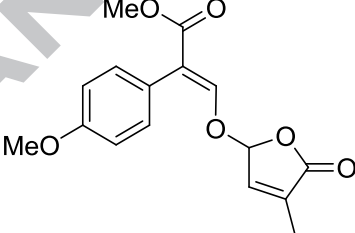
TSK16	$C_{12}H_8BrNO_3$; 294.10	
TIT3	$C_{15}H_{14}O_5$; 274.27	
TIT7	$C_{15}H_{13}ClO_5$; 308.71	
TIT14	$C_{16}H_{16}O_6$; 304.29	

Table 1: Structure and molecular weight of synthetic Strigolactone analogues.

Initially, we screened seven newly synthesized SLs (Table 1) on HepG2 cells to evaluate their anti-proliferative activity. Compounds **TSK13**, **TSK14**, **TSK15**, **TSK16**, **TIT3**, **TIT7** and **TIT14** reduced HepG2 cell viability by about 43.5%, 40.7%, 39%, 43.8%, 63.4%, 61.4% and 50.1% respectively at 80 μ M (Fig 1A). This effect started to be significant at 40 μ M for **TSK13**, **TIT7** and **TIT14** respectively. The corresponding IC₅₀ values for the tested SLs were calculated as mentioned in Table 2.

Table 2: IC₅₀ Values of SLs analogues.

SL Analogues	IC ₅₀ (μM)
TSK13	85.85 ± 1.2
TSK14	89.45 ± 2.4
TSK15	90.33 ± 1.9
TSK16	87.59 ± 2.1
TIT3	63.46 ± 1.4
TIT7	67.81 ± 1.8
TIT14	82.12 ± 0.9

The most effective anticancer SLs **TIT3**, **TIT7** and **TIT14** were tested further on the normal baby hamster kidney cells (BHK cells) to confirm their selectivity towards cancer cells (tested at similar concentrations used for HepG2) (Fig. 1B). Similar to its anti-proliferative activities towards cancer cells, **TIT14** also has some inhibitory effects on BHK (reduction of around 33% in cell viability) in comparison to **TIT3** and **TIT7** (Fig. 1B). Interestingly, both **TIT3** and **TIT7** had less inhibition of BHK cell viability after incubation of BHK with **TIT3** and **TIT7** for a time period of 48 h (Fig 1B). At the highest concentration (80μM), **TIT3** and **TIT7** inhibited BHK cell viability only by 18% and 23% respectively, while with the same concentration **TIT3** and **TIT7** inhibited HepG2 cell viability by 73% and 61% respectively just

after 24 h (Fig. 1A). These findings suggest that **TIT14** showed more cytotoxicity relative to **TIT3** and **TIT7** towards BHK cells.

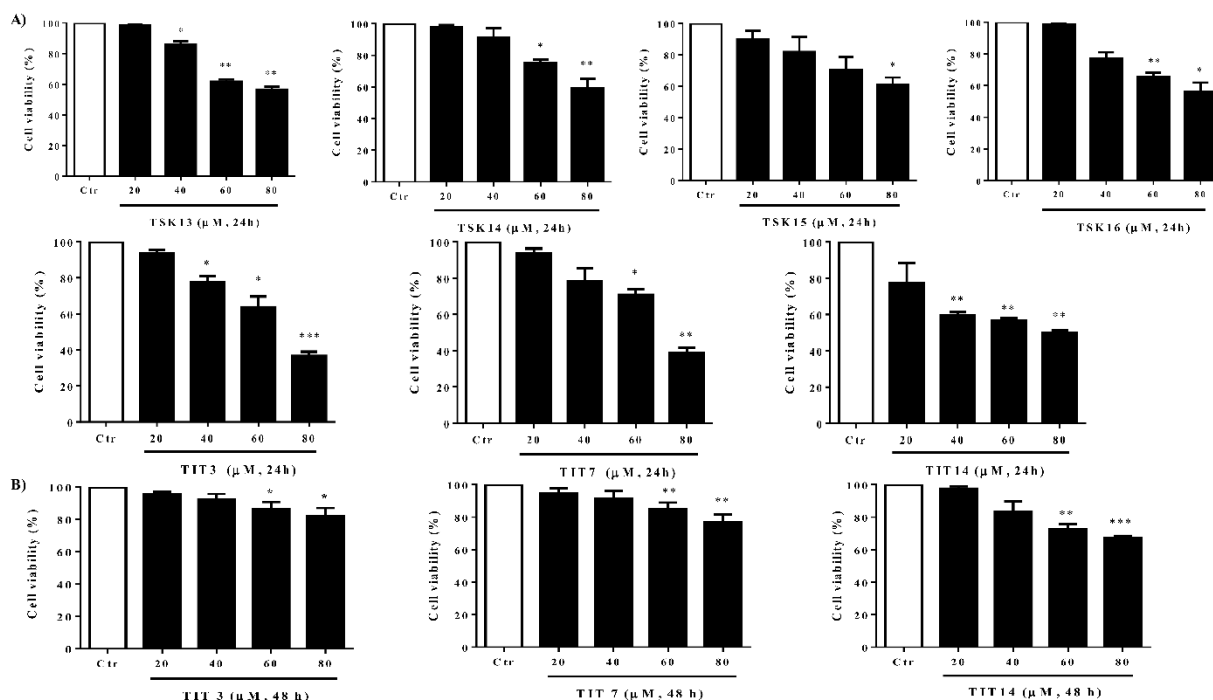


Fig 1: (A) Dose-dependent antiproliferative effect of SL analogues on cell viability of HepG2. HepG2 cells were treated with several concentrations of SLs for 24 h. Cell viability was assessed using WST-1 assay as described in supporting information. The values here are represented as mean \pm S.E.M. of two independent experiments in triplicates; statistically significant (unpaired 't' test; two-tailed): *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$; versus the corresponding untreated control. **(B) Dose-dependent effect of SL analogues TIT3, TIT7 and TIT14 on cell viability of normal BHK cells.** BHK cells were treated with several concentrations of SLs for 24 h. Cell viability was assessed using WST-1 assay as described in supporting information. The values here are represented as mean \pm S.E.M. of three independent experiments in triplicate; statistically significant (unpaired 't' test; two-tailed): *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$; versus the corresponding untreated control.

To determine whether SLs **TIT3** and **TIT7** induce inhibitory effects in another type of solid tumor besides HepG2 cells, we performed experiments with the human prostate cancer cell line, PC3. Interestingly, both **TIT3** and **TIT7** significantly decreased cell viability after 24 h treatment as shown in the Fig. 2A & B at a concentration of 40 μ M. Additionally, we evaluated their anti-proliferative effect on T-cell acute lymphoblastic leukemia cell line, Jurkat. We observed that the cell viability of JK was reduced in a dose-dependent manner (Fig. 2C, D) similar to HepG2 and PC3 cell lines. Indeed, the reduction in cell viability started to be significant ($P < 0.05$) starting from 40 μ M for **TIT3** (Fig. 2C) and 20 μ M for **TIT7** (Fig. 2D). The corresponding IC_{50} values for the tested SLs on PC3 and Jurkat cells were calculated as mentioned in Table 3. These results indicate that SLs **TIT3** and **TIT7** can inhibit cell proliferation in both solid and hematological tumors.

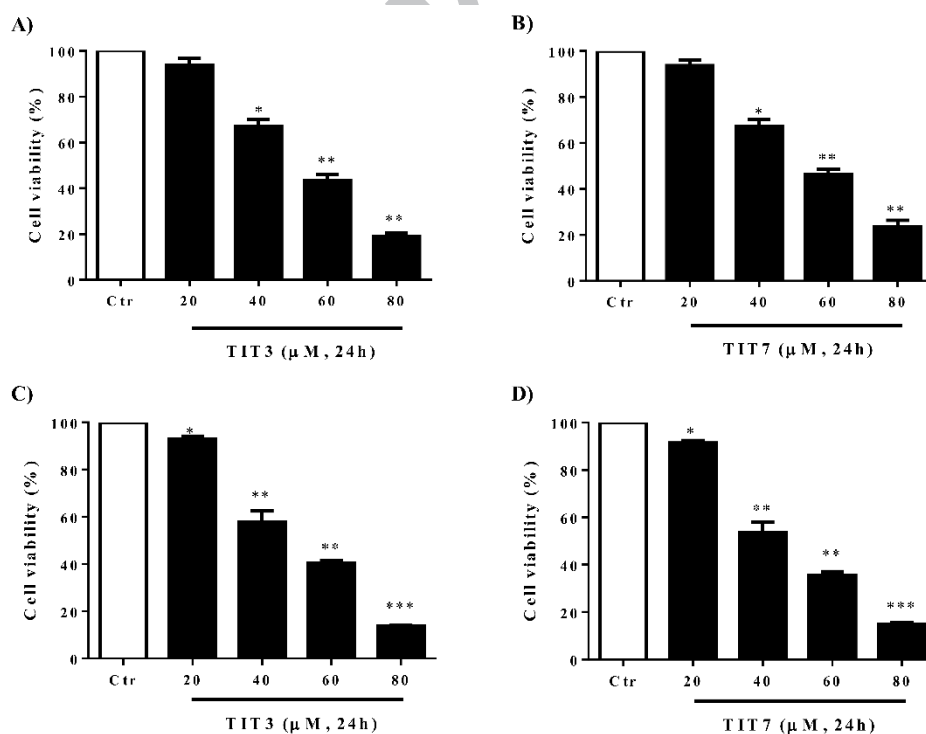


Fig 2: Dose-dependent antiproliferative effect of SL analogues on cell viability of PC3 and Jurkat cells. PC3 (A, B) and Jurkat cells (C, D) were treated with several concentrations of **TIT3** and **TIT7** for 24 h. Cell viability was assessed using WST-1 assay as described in

supporting information. The values here are represented as mean \pm S.E.M. of two independent experiments in triplicates; statistically significant (unpaired 't' test; two-tailed): *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$; versus the corresponding untreated control.

SL Analogues	IC ₅₀ (μ M) PC3	IC ₅₀ (μ M) JURKAT
TIT3	55.07 \pm 1.7	46.36 \pm 2.4
TIT7	56.64 \pm 1.1	44.71 \pm 0.8

Table 3: IC₅₀ Values of SLs analogues TIT 3 AND TIT 7 on PC3 and JURKAT cells

Thereafter, **TIT3** and **TIT7** were selected for further cytotoxicity studies on HepG2. The time-dependent effect of both compounds was then investigated at 80 μ M for different time periods (1, 4, 8 and 12 h). Treatment of HepG2 with 80 μ M of **TIT3** and **TIT7** induced a time-dependent decrease in HepG2 cell viability, this effect was prominent at 13% and 11% in the first 4 h and significantly reached 20% and 19% after 8 h for **TIT3** and **TIT7** respectively (Fig 3A and B). The inhibition of HepG2 cell viability was more pronounced at 12 h by 31% for **TIT3** compared to 26% for **TIT7** (Fig. 3 A and B).

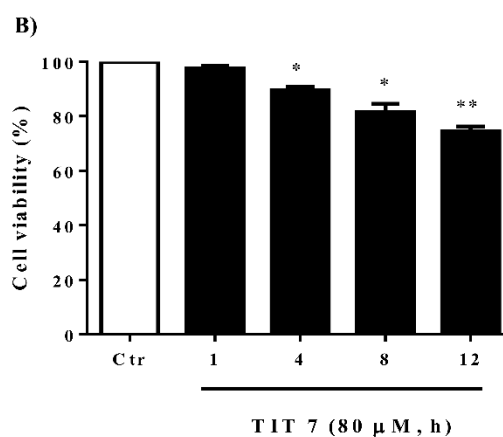
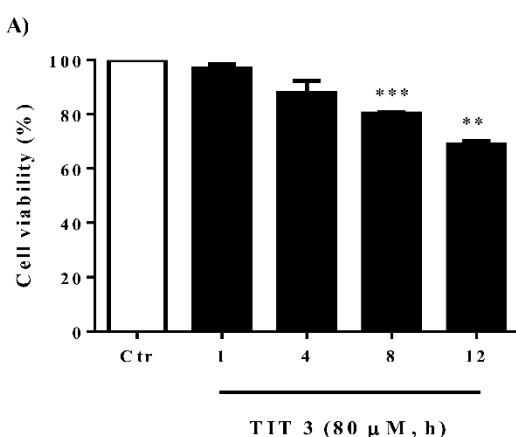
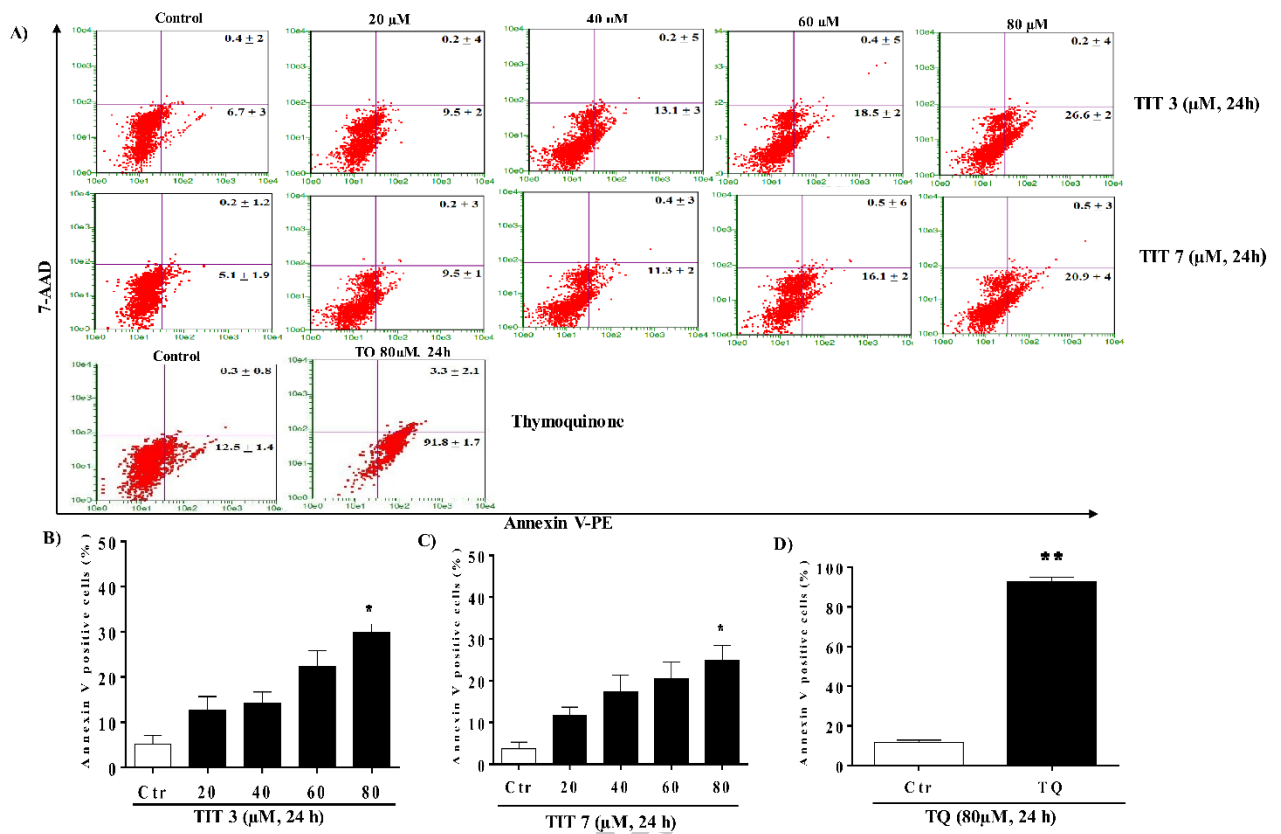


Fig 3: Time-dependent effect of SL analogues TIT3 and TIT7 on cell viability of HepG2.

HepG2 cells were treated using 80 μ M with **TIT3** (A) and **TIT7** (B) for indicated time durations. The values represented here are mean \pm S.E.M of two independent experiments in triplicate; statistically significant (unpaired 't' test; two-tailed): *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$; versus the corresponding untreated control.

To determine the synthetic SLs analogues-induced mechanism involved in the inhibition of HepG2 cell viability, we analyzed the effect of **TIT3**, **TIT7** and the anticancer drug-thymoquinone (**TQ**) as a positive control on the apoptosis. AnnexinV-7AAD staining was performed with different concentrations for 24 h (Fig 4A). The results showed that both **TIT3** and **TIT7** induced apoptosis in HepG2 cells, with the pro-apoptotic effect being approximately at 12.7% and 11.7% annexin V positive cells at a concentration of 20 μ M. However, with a concentration of 40 μ M, the annexin V positive cells were 14.2% and 17.3% for SLs analogues **TIT3** and **TIT7** respectively (Fig. 4B and C). At 60 μ M, there was an increase in the number of apoptotic cells nearly 22.5% and 20.5% and reached significantly up to 29.8% and 24.9% at the maximum concentration of 80 μ M for **TIT3** and **TIT7** respectively (Fig 4B and C). A time-dependent annexin V/7AAD staining was then performed to study the chronology of events induced by **TIT3** and **TIT7**. Exposure of HepG2 cells to 80 μ M of **TIT3** and **TIT7** induced a significant increase in the number of apoptosis cells from 6 h by about 15.5% and 14.5% for **TIT3** and **TIT7** respectively (Fig 5A, B and C).

Fig 4: Dose-dependent Annexin V/7AAD demonstrates pro-apoptotic effect of SL analogues TIT3 and TIT7 in HepG2. HepG2 cells were treated with several concentrations of **TIT3** (A upper panel and B) and **TIT7** (A middle panel and C) or **TQ** as a positive control (A lower panel and D) for 24 h. Values shown here are mean \pm S.E.M of two independent experiments in triplicates; statistically significant (unpaired 't' test, two-tailed): *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$.



p<0.001 versus the corresponding untreated control.

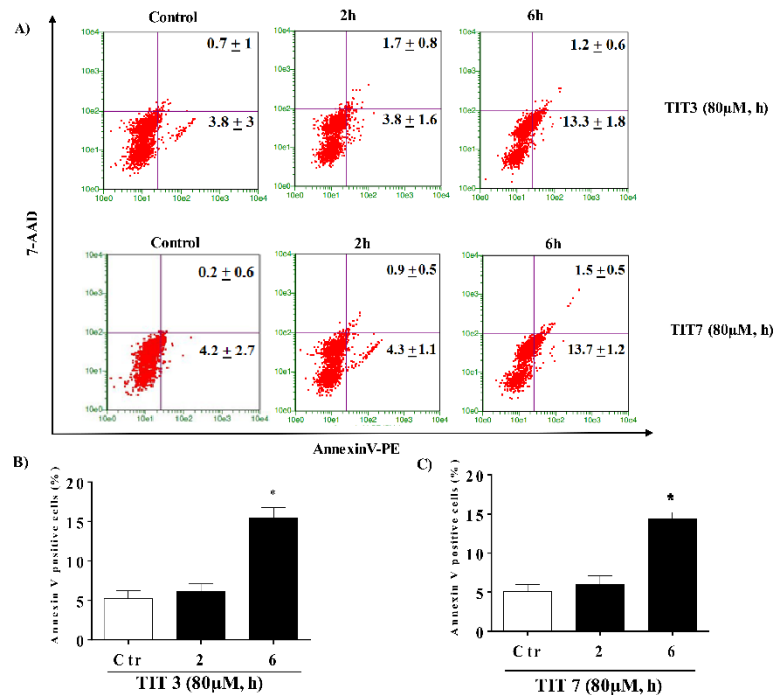


Fig 5: Time-dependent Annexin V/7AAD staining of SL analogues TIT3 and TIT7 reveals pro-apoptotic activity in HepG2. HepG2 cells were treated for 2 and 6 h with 80 μ M of **TIT3** (A upper panel and B) and **TIT7** (A lower panel and C). Values shown here are mean \pm S.E.M. of two independent experiments in triplicate; statistically significant (unpaired 't' test, two-tailed): *, $p < 0.05$ versus the corresponding untreated control.

Altogether, these findings indicate that SLs-induced inhibition of HepG2 cell viability could result from apoptosis process, but other cell death mechanisms could be involved as there were variations in results of cell viability and apoptotic assays.

To determine whether **TIT3** and **TIT7** inhibit liver cancer cell migration, a wound healing assay was performed. The treatment of HepG2 cells with **TIT3** and **TIT7** at a concentration of 80 μ M, markedly inhibited the migration of treated cells compared to untreated ones (Fig. 6A). The results show that the scratch on the confluent monolayer of HepG2 was visibly filled again with the cells after a time period of 24 h, but the cells treated with **TIT3** and **TIT7** didn't show any such migration on the scratched area when observed under the microscope (Fig 6B & C) after the same incubation period. Thus, these results indicate that analogues **TIT3** and **TIT7** inhibit the migration of invasive cells suggesting that these analogues may interfere with the microtubular network of HepG2 cells, and hence check the metastasis of tumors.

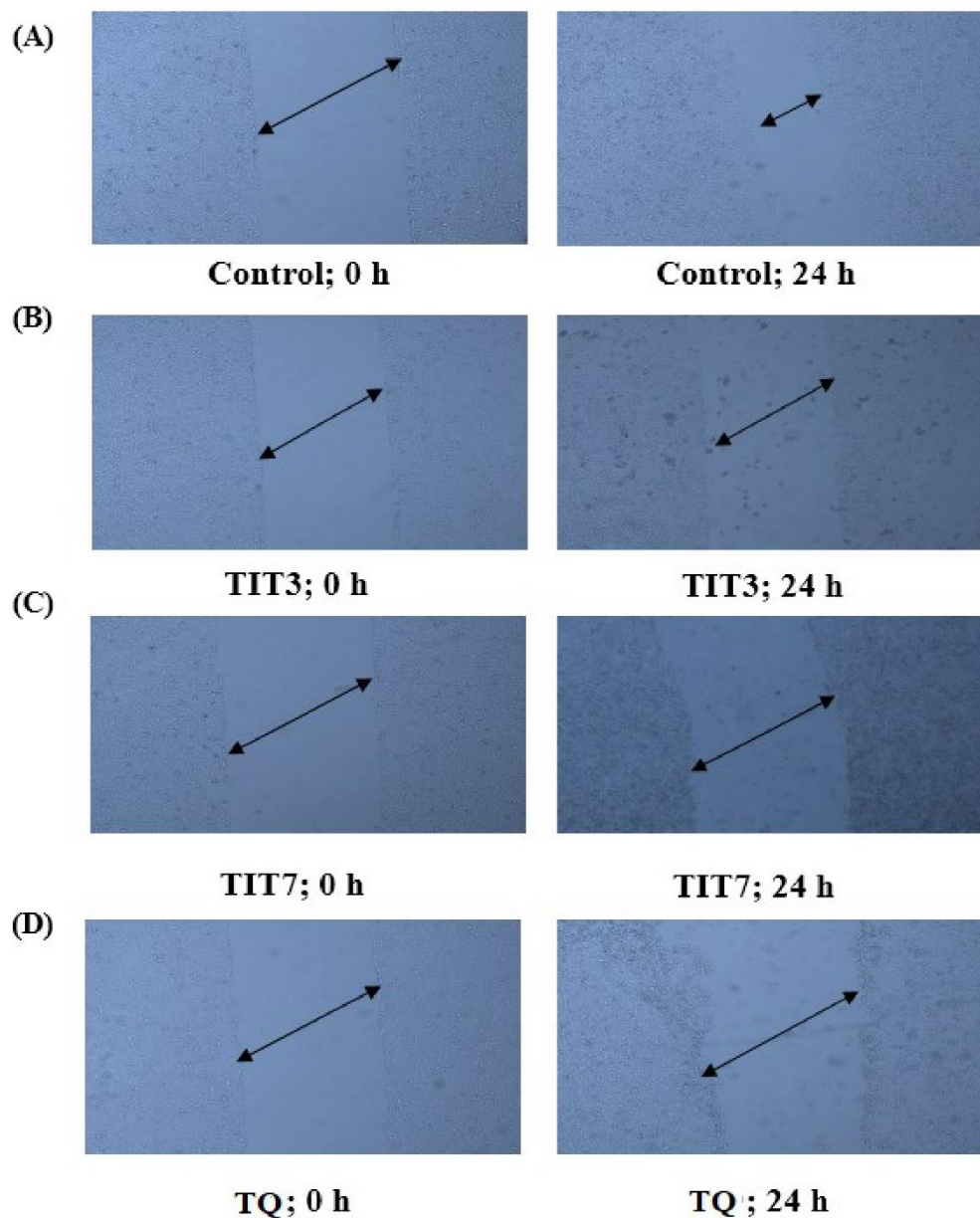


Fig 6: TIT3 and TIT7 inhibited the migration of HepG2 cells. The control shows cellular proliferation after 24 h, as the wound has been “healed” to an extent, the scratch is more prominent at 0 h (A). SL analogues **TIT3** (B) and **TIT7** (C) treated cells, apparently do not show any signs of cellular proliferation or migration to the scratched area of the confluent monolayer of HepG2 after 24 h. **TQ** (positive control) shows maximum cellular inhibition after 24 h with a slight increase in the scratched area (D).

Different SLs analogues have been shown to exert inhibitory effects on a panel of cancer cells, including those of leukaemia, lung, prostate, breast and bone cancers²² suggesting them as potent anti-cancerous agents. The present study indicates that SLs analogues **TIT3** and **TIT7** decreased cell viability of HepG2 cells by dose- and time-dependent mechanism. While **TIT14** inhibited both normal baby hamster kidney cells (BHK cells) and cancer cells (HepG2 and JK cell lines), the two SLs analogues **TIT3** and **TIT7** had significant inhibitory effects only on HepG2 and JK cells. Moreover, such inhibitory effect was not observed in **TIT3**- or **TIT7**-treated BHK cells even after 48 h of treatment, suggesting them as promising selective anticancer agents. This study also showed that **TIT3** and **TIT7** induced apoptosis in HepG2 cells by dose- and time-dependent manner. Additionally, this data indicates that SLs analogues **TIT3** and **TIT7** inhibited the migration of invasive HepG2 cells.

Moreover, it is noteworthy that till date, a proper conclusion and perception of the targets and molecular effects of SLs analogues on cancer cells is quite limited.²⁵ Our results suggest that there is a high correlation between the inhibitory activities of SLs on HepG2 cells and their effects on microtubules. The present study shows that compounds **TIT3** and **TIT7** may interfere with the microtubule network of HepG2 cells, and hence in the metastasis of tumors. This is not entirely surprising considering that SLs analogues have been reported to exert inhibitory effects on microtubules of cancer cells.²⁶ Indeed, SLs analogues have been suggested to inhibit the migration of MDA-MB- 231-a highly invasive breast cancer cell line by affecting the integrity of the microtubular network.²⁶ Apart from **TIT3** and **TIT7**, the **TQ** used as a positive control in this study also inhibited the migration of invasive HepG2 cells. Interestingly, microtubule network of lung cancer-A549 cells²⁷ and human astrocytoma cells (cell line U87)²⁸ have been

reported as a primary target of **TQ**. Indeed, **TQ** depolymerized the microtubular network of A549 cells with subsequent apoptosis²⁷ and targeted the structure of microtubules directly rather than the modulation of its dynamics.²⁸ The present study showed that **TQ** was able to inhibit the migration of HepG2 in the same way like **TIT3** and **TIT7** supporting the idea that these analogues exert their inhibitory effects on HepG2 through targeting microtubules.

Unanticipatedly, the apoptotic effect of these analogues was not similar to the effect shown by the same analogues in cellular viability (WST-1) assay. This dissimilarity might be due to the stimulation of some other cell death mechanisms in addition to apoptosis. Previously reported studies on prostate, lung, colon, breast cancer cell lines^{22,23} and osteosarcoma cell line²⁵ established apoptosis as their primary cell death mechanism, this study might point towards HepG2 cells simultaneously triggering other cell death mechanisms along with apoptosis when treated with SLs analogues. Nevertheless, the number of cells undergoing apoptosis did increase with an increase in the dosage patterns of both **TIT3** and **TIT7** which is quite evident from the figures. The increase was more pronounced with the increase in the time duration with both the analogues, showing a significant increase after 6 h.

Our results show that **TIT3** and **TIT7** inhibited the proliferation of HepG2 cells and induced apoptosis. Considering the fact that **TIT3** and **TIT7** inhibited the migration of HepG2 cells, SLs analogues-induced apoptosis could result from the modulation of the microtubular network. In the same context, SLs analogues have been shown to induce apoptosis in breast cancer²⁶ and in a panel of diverse cancer cell lines including melanoma, colon, lung, prostate, osteosarcoma and leukaemia cells²² through targeting of microtubules. Our results also showed that **TIT3** and **TIT7** had minimal inhibitory effects on healthy cells compared to either hepatocellular carcinoma, prostate and leukemia cancer cells suggesting them as selective

promising anticancer agents. Our study confirms the hypothesis that SLs analogues have shown low inhibitory effects on growth and survival of several normal cells ²². This finding also suggests that there might be unknown yet distinctive receptors for SLs on cancer cells, attributing to the selectivity of cancer cells with SLs analogues which requires further investigation ²⁵.

In conclusion, to the best of our knowledge, this is the first ever study performed to evaluate the anti-proliferative activity of SLs analogues on HepG2 and JK cells. The SLs analogues **TIT3** and **TIT7** inhibited the growth of HepG2 and JK cells. It would be of interest to know whether SLs **TIT3** and **TIT7** target microtubules dynamics directly or the expression of its tubulin structure. Both **TIT3** and **TIT7** showed relatively lower toxicity towards normal cells and high efficacy against cancer cells suggesting their usage as promising anticancer agents to reduce chemotherapy-related toxicity while promoting the anticancer activity.

Conflict of interest:

The authors declare that they have no financial conflict of interest.

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