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RESEARCH ARTICLE

In Vitro Effects of Salvinorina on mRNA Expression of Liver

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Abstract

Salvinorin A (SA) is the main bioactive compound of Salvia divinorum (S. divinorum) and is the responsible for the hallucinogenic properties of this plant. Although the main known biological effects of SA, and S. divinorum, occur in the Central Nervous System (CNS), its effects on the hepatic system, where it is largely metabolized by various enzymes, in particular, cytochrome P450 family (CYP450), carboxylesterases (Ces) and Uridine Diphosphate Glucuronosyl Transferases (UGTs), are still largely unknown.

With this in vitro study, we intended to screen the mRNA expression of some relevant genes both for this compound and/or other molecules in two different hepatic cell lines (WRL68 and HepG2).

We evaluated the toxicity of SA in each cell line after an exposure to some concentrations of SA during various periods of time and tested the putative role of SA in the regulation of mRNA expression of apolipoprotein B100 (ApoB100), carboxylesterase 1 (Ces1), cytochrome C (Cyt. C), cytochrome P450 1A2 (CYP1A2), cytochrome P450 2D6 (CYP2D6), cytochrome P450 3A4 (CYP3A4) and uridine diphosphate glucuronosyltransferase 1A1 (UGT 1A1) genes. The cytotoxicity of SA was accessed by MTT assays and the mRNA expression was evaluated by real-time PCR.

Data showed that 50 μM of SA significantly decreased the Cellular Viability (CV) in both cell lines. Also, 10μM of the compound was found to promote a significant decrease in CV in HepG2 cells, but only for longer periods of exposure to SA. Furthermore, in cell sex posed to 1 μM of SA it was observed a significant alteration in mRNA expression of the genes in a cell- and time-dependent manner. Additionally, it was also found that Ces 1 is not expressed

in WRL 68 cells as its mRNA was not detected.

Overall, our findings suggest that SA has a low toxicological profile but it should be monitored when consumed continuously at higher doses or when combined with Apo B100, Ces 1, Cyt C, CYP 1A2, CYP 2D6, CYP 3A4 and UGT 1A1 target compounds.

Introduction

Salvia divinorum (S. divinorum) is a hallucinogenic plant native from Oaxaca, México, commonly used by Mazatecs [1]. Many substances were isolated from the plant: salvinorin A (SA) to J [2-5], divinatorin A to F [3,5], salvidivin A to D [3], salvinicin A to B [6]. SA is a neoclerodanediterpen and is the main bioactive compound responsible for the hallucinogenic properties of S. divinorum [2,7]. To reachintense hallucinogenic effects, the most effective form of consumption is through oral absorption in doses ranging from 200 to 500 µg of SA or through its vaporization and inhalation [7]. SA has a rapid onset and reaches the Central Nervous Systems (CNS) very quickly due to its high lipophilicity and low molecular weight, which facilitate its crossing through the brain blood barrier and distribution within the brain, in higher levels in the cerebellum and visual cortex [8,9].

Structurally, SA differs from all known hallucinogens, as it is the first naturally occurring compound with high selectivity for kappa opioid receptors (KOR), a target of psychotropic compounds [10], which belong to opioid system and which are widely expressed in the peripheral and CNS [11]. SA has a fast distribution witha half-life time ($t_{1/2}$)of 56.6 min [12] and is metabolized by various enzymes including cytochrome P450 (CYP450) [8], carboxylesterases (Ces) [13] and uridine diphosphate glucuronosyltransferases (UGTs) [8].

Table 1: Primer sequences of human apolipoproteinB100 (hApo B100), carboxyelesterase 1 (hCes 1), cytochrome P450 1A2 (hCYP 1A2), 2D6 (hCYP 2D6), 3A4 (hCYP 3A4), cytochrome C (hCyt C) and uridine diphosphate glucuronosyltransferases 1A1 (hUGT 1A1) in real-time PCR experiments.

Primer Designation	Sequence 5'- 3'
hApoB100 fw	atccacatc acct cc aaagc
hApoB100 rv	ctgaggctgtcc aca ctg aa
hCes1 fw	gcatctggg gat tcttcagc
hCes1 rv	ttctcctcccgctgactctc
hCYP 1A2 fw	ctgggc act tcgaccctt ac
hCYP 1A2 rv	gcaggaac caca ggaacctc
hCYP 3A4 fw	ccgagtggatttccttcagc
hCYP 3A4 rv	aggtgg gtg gtgcct tat tg
hCYP 2D6 fw	tctcttggacaaagccgtga
hCYP 2D6 rv	gctgggatatgcaggagg ac
hCytCfw	cct cag gga gag gtg gcttt
hCytCrv	agcccaagcaaa gag gga ac
hGAPDHfw	cgc cag ccgagccacatc
hGAPDHrv	cgccca ata cgaccaaatccg
hUGT1A1 fw	ccttgcctcagaattccttc
hUGT1A1 rv	att gat ccc aaa gag aaaacc ac

Previous studies showed that SA has a great potential and a safe physiological profile for therapeutic usage, prevents drug addiction [14-18], acts in various neurologic disorders [19-22], treats some gastrointestinal issues [23,24] and presents antinociceptive [25,26] and anti-inflammatory properties [26,27].

During the last few years, the SA demand has increased, in particular in the youth, due to its easy acquisition and its potent hallucinogenic effects.

Nevertheless, its biological effects are poorly understood and almost completely related with its effects in the CNS. However, the hepatic system plays a major role

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Table 2: Reaction conditions used for each gene (ApoB100, Ces1, CYP 1A2, CYP 2D6, CYP 3A4, CytC, GAPDH and UGT1A1) in each cell line (HepG2 and WRL 68).

Gene	Reaction conditions
	HepG2
ApoB100	10 µL SYBR Green, 3 pmolprimerFw and Rv; 1 µL cDNA (1:1)
Ces1	10 µL SYBR Green, 3 pmolprimerFw and Rv; 1,5 µL cDNA (1:1)
CYP 1A2	10 µL SYBR Green, 2,5 pmolprimerFw and Rv; 1 µL cDNA (1:1)
CYP 2D6	10 µL SYBR Green, 3 pmolprimerFw and Rv; 1 µL cDNA (1:1)
CYP 3A4	10 µL SYBR Green, 3 pmolprimerFw and Rv; 1 µL cDNA (1:1)
Cyt C	10 µL SYBR Green, 2 pmolprimerFw and Rv; 1 µL cDNA (1:1)
GAPDH	10 µL SYBR Green, 3 pmolprimerFw and Rv; 1 µL cDNA (1:1)
UGT 1A1	10 µL SYBR Green, 5 pmolprimerFw and Rv; 1,5 µL cDNA (1:1)
	WRL68
ApoB100	10 µL SYBR Green, 4 pmolprimerFw and Rv; 1 µL cDNA (1:1)
Ces1	10 µL SYBR Green, 3 pmolprimerFw and Rv; 1,5 µL cDNA (1:1)
CYP 1A2	10 µL SYBR Green, 4 pmolprimerFw and Rv; 1 µL cDNA (1:1)
CYP 2D6	10 µL SYBR Green, 3 pmolprimerFw and Rv; 1 µL cDNA (1:1)
CYP 3A4	10 µL SYBR Green, 4 pmolprimerFw and Rv; 1,5 µL cDNA (1:1)
Cyt C	10 µL SYBR Green, 3 pmolprimerFw and Rv; 1 µL cDNA (1:1)
GAPDH	10 µL SYBR Green, 3 pmolprimerFw and Rv; 1 µL cDNA (1:1)
UGT 1A1	10 µL SYBR Green, 5 pmolprimerFw and Rv; 1 µL cDNA (1:1)

in humans [28], SAis metabolized by some enzymes expressed in liver and there is no data regarding its effects on the liver expression of apolipoprotein B100 (ApoB100), carboxylesterase 1 (Ces1), CYP450 1A2 (CYP1A2), CYP4502D6 (CYP2D6), CYP4503A4 (CYP3A4), cytochrome C (Cyt C) and UGT 1A1 (UGT1A1) genes. In fact, the structure of SA reveals that it could regulate, and be metabolized, by certain enzymes, including Ces 1, CYP 1A2, CYP 3A4, UGT 1A1 and CYP 2D6 [8].

Apo B100 is a protein that regulates the tissue uptake of low density lipoproteins (LDL) in plasma [29], and it is related with some genetic disorders as familial hypercholesterolemia and familial defective ApoB100 [30].

Cyt C is a free hemeprotein, pivotalfor the electron transport chain, and it also plays a role in apoptosis when released from the mitochondria [31].

Ces1 enzyme is mainly expressed in the liver where it has a pivotal role in the metabolism of xenobiotics and other compounds [32].

The CYP450 family includes several mono oxygenase enzymes mostly involved in human drug metabolism [33]. These enzymes are mainly responsible for the phase I oxidative metabolism and 90% of human drugs are metabolized by six main cytochrome isoforms: CYP 1A2, 2C9, 2C19, 2D6, 2E1 and 3A4 [34]. Furthermore, some substrates are activated following CYP450 metabolism which could generate toxic compounds [35]. The isoform 1A2 is induced by cigarette smoking and is also involved in carcinogen bio activation [33]. The CYP 3A4 is one of the main human CYP450 enzymes as it hasa major role in drug metabolism, being there sponsible for the metabolism of more than 50% of xenobiotics and drugs [36-38]. The CYP 2D6 is mainly expressed in the liver and it is also involved in drug metabolism [33], including anti-arrhythmic, antidepressant, anesthetic and anal gesicdrugs [34].

The UGTs are glucuronidation enzymes involved in the clearance and detoxification of xenobiotics [37,38]. The isoform UGT1A1 is the most studied form due to its genetic variability and relevance in the bilirubin clearance [39].

Taken together, with this in vitro study we intended to reveal the putative physiological relevance of SA consumption in the hepatic system and to figure out which are the possible impact of S. divinorum consumption to human health. Thus, the aim of this study is to study the in vitro SA toxicity in two hepatic cell lines, and to ascertain its effects on the expression of certain genes related with drug metabolism.

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Table 3: Amplification conditions used for each gene (ApoB100, Ces1, CYP 1A2, CYP 2D6, CYP 3A4, Cyt C, GAPDH and UGT1A1) in each cell line (HepG2 and WRL 68).

Gene	Amplification conditions
	HepG2
ApoB100	3 min at 95 ºC; 40 cycles (15 sec at 95ºC; 30 sec at 63 ºC; 30 sec at 72 ºC)
Ces1	3 min at 95 ºC; 40 cycles (15 sec at 95ºC; 30 sec at 64 ºC; 30 sec at 72 ºC)
CYP 1A2	3 min at 95 ºC; 40 cycles (15 sec at 95ºC; 30 sec at 63 ºC; 30 sec at 72 ºC)
CYP 2D6	3 min at 95 ºC; 40 cycles (15 sec at 95ºC; 30 sec at 63 ºC; 30 sec at 72 ºC)
CYP 3A4	3 min at 95 ºC; 45 cycles (15 sec at 95ºC; 30 sec at 63 ºC; 30 sec at 72 ºC)
Cyt C	3 min at 95 ºC; 40 cycles (15 sec at 95ºC; 30 sec at 63 ºC; 30 sec at 72 ºC)
GAPDH	3 min at 95 ºC; 40 cycles (15 sec at 95ºC; 30 sec at 63 ºC; 30 sec at 72 ºC)
UGT 1A1	3 min at 95 ºC; 45 cycles (15 sec at 95ºC; 30 sec at 60 ºC; 30 sec at 72 ºC)
	WRL68
ApoB100	3 min at 95 ºC; 45 cycles (15 sec at 95ºC; 30 sec at 60 ºC; 30 sec at 72 ºC)
Ces1	3 min at 95 ºC; 45 cycles (15 sec at 95ºC; 30 sec at 63 ºC; 30 sec at 72 ºC)
CYP 1A2	3 min at 95 ºC; 40 cycles (15 sec at 95ºC; 30 sec at 63 ºC; 30 sec at 72 ºC)
CYP 2D6	3 min at 95 ºC; 40 cycles (15 sec at 95ºC; 30 sec at 63 ºC; 30 sec at 72 ºC)
CYP 3A4	3 min at 95 ºC; 45 cycles (15 sec at 95ºC; 30 sec at 63 ºC; 30 sec at 72 ºC)
Cyt C	3 min at 95 ºC; 40 cycles (15 sec at 95ºC; 30 sec at 63 ºC; 30 sec at 72 ºC)
GAPDH	3 min at 95 ºC; 40 cycles (15 sec at 95ºC; 30 sec at 63 ºC; 30 sec at 72 ºC)
UGT 1A1	3 min at 95 ºC; 45 cycles (15 sec at 95ºC; 30 sec at 60 ºC; 30 sec at 72 ºC)

Material and Methods

Salvinorin a stock solution

One milligram (1mg) of SA (SA; Sigma Aldrich, Inc.)was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Inc.) and 0.1% HCl 0.1 M (Merck Millipore).

Cell cultures

The two cell lines used in this study were HepG2 (human hepatocellular carcinoma cells) and WRL68 (human fetal hepatic cells).

HepG2 and WRL68 cell lines were obtained from American Type Culture Collection (ATCC) and where cultured in 75 or 175 cm^2 culture flasks in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Inc.), 100 U/mL penicillin (Sigma-Aldrich, Inc.) and 100 μg/mL streptomycin (Sigma-Aldrich, Inc.) and maintained in a humidified air incubator at 37 ºC 5% CO_2.

For both cell lines, the medium was renewed every 2-3 days until cells reached confluence.

Cell treatments

When cultures reached approximately 90-95% confluence, cells were tripsinizedfrom the cultured flasks, quantified by the trypan-blue assay and seeded in quadruplicated 96-well culture plates (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromideassays- MTT assay) and duplicated in 6-well culture plates (real-time PCR assays – RT-PCR assay). HepG2 and WRL68 were seeded at 2,5 x 10^4 (MTT) and 4 x 10^5 cells/well (real-time PCR)and left to adhere and to grow in appropriate medium. After reaching 85-90% confluence the medium was substituted for the correspondent serum-free medium and left for 12h. After this period, cells were incubated with SA, in appropriate serum-free medium, at 0, 0.1, 1, 10 and 50 μM for 12, 24,

36, 48 and 72h for MTT assays and for RT-PCR at 0 and 1 for 12, 24 and 72h.The DMSO was always kept below 0.1% to prevent the cytotoxicity induced by this solvent. For all assays each experiment was repeated in triplicate and in three independent experiments.

MTT cell viability assay

In vitro cytotoxicity was evaluated by MTT assay (Sigma-Aldrich, Inc.) as described previously [40]. Briefly, after the incubation of cells with each concentration for 12, 24, 36, 48 or 72h, the medium was removed and 200 μL of phosphate buffer saline (PBS) were added to wash the cells. Then, 100 μL of MTT solution (0.5 mg/mL) in serum-free correspondent medium were added to cells, in the dark, for 60-120 min at 37 ºC. After, the MTT-containing solution was removed from wells and the formazan crystals were dissolved in 40 m MHCl solution in isopropanol. After a 5 min of incubation in the, the solution was transferred to a new multi well plate and the absorbance was measure dat 570 nm using a micro plate reader EZ Read 400 (Biochrom). Each experiment was performed in triplicate and repeated in three independent experiments.

Total mRNA extraction and real-time PCR

Total mRNA extraction was performed according to described by Martinho et al. (2012) [41] with minor modifications. Briefly, total mRNA was extracted from cells using TRI Reagent (Sigma-Aldrich, Inc), following the manufacturer's instructions. Total mRNA of each sample was quantified using Nanophotometer (Implen, Munich, Germany) and 1 μg was reverse transcribed for 1h at 37ºC in a 20μL reaction solution containing 10X RT Buffer (Nzytech), 25 pmol of random hexamer mix (Nzytech), 0.5 mM of each dNTP (dATP,dCTP, dGTP and dTTP; Nzytech), 0.1 M dithiothreitol (DTT) and 200 U NZY reverse transcriptase (Nzytech).

The mRNA levels of ApoB100, Ces1, CYP 1A2, 2D6, 3A4, CytC and UGT 1A1in HepG2 and WRL 68 cells were analyzed by real-timePCR in cells incubated with 0 and 1 of SA for 12, 24 and 72h. The human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as endogenous control gene. Sequences of all PCR primers used are indicated in Table (1).

To analyze the mRNA expression of genes in both cell lines, reactions were carried out using the conditions indicated in Table (2) in a 20 μL-reaction tube. The amplification conditions used for each cell line and for each gene are mentioned in the Table (3).

The amplified PCR fragments were checked by melting curves: reactions were heated from 55 to 95 ºC with 10 s hold at each decreasing cDNA concentrations (1:1; 1:10; 1:100 and 1:1000) and the reaction efficiencies were calculated. Real-time PCR was carried out using the iCycler IQ System (Bio-Rad) and fluorescence was measured after each cycle. Every reaction was done, at minimum, in triplicate and each experiment was performed in three independent times.

Statistical analysis

The percentage of cellular viability relative to controls (CV) was determined and expressed as mean ± standard deviation, using the formula:

CV = (Average of the absorbance of quadruplicate treated cells / Average of the absorbance of control cells) x 100 %

Data obtained was compared by means of a two-tailed unpaired Student´s t test and were considered statistically significant for a p-value lower than 0.05 ($p < 0.05$).

The data collected from real-time PCR experiments were analyzed using the mathematical model proposed

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by Pfaff: $2^{-(\Delta\Delta Ct)}$ [42], which allows the determination of differences in expression levels between control and treated cells, taking into account reaction efficiency and reference gene normalization.

Results

Evaluation of SA-mediated Cyto toxicity

This study aimed to evaluate the in vitro toxicity of SA and to compare it in the two hepatic cell lines. So assess it, MTT assays were performed and the relative cellular viability (CV) in HepG2 (Figure 1) and WRL68 (Figure 2) cells after incubation with SA for 12, 24, 36, 48 and 72h were determined.

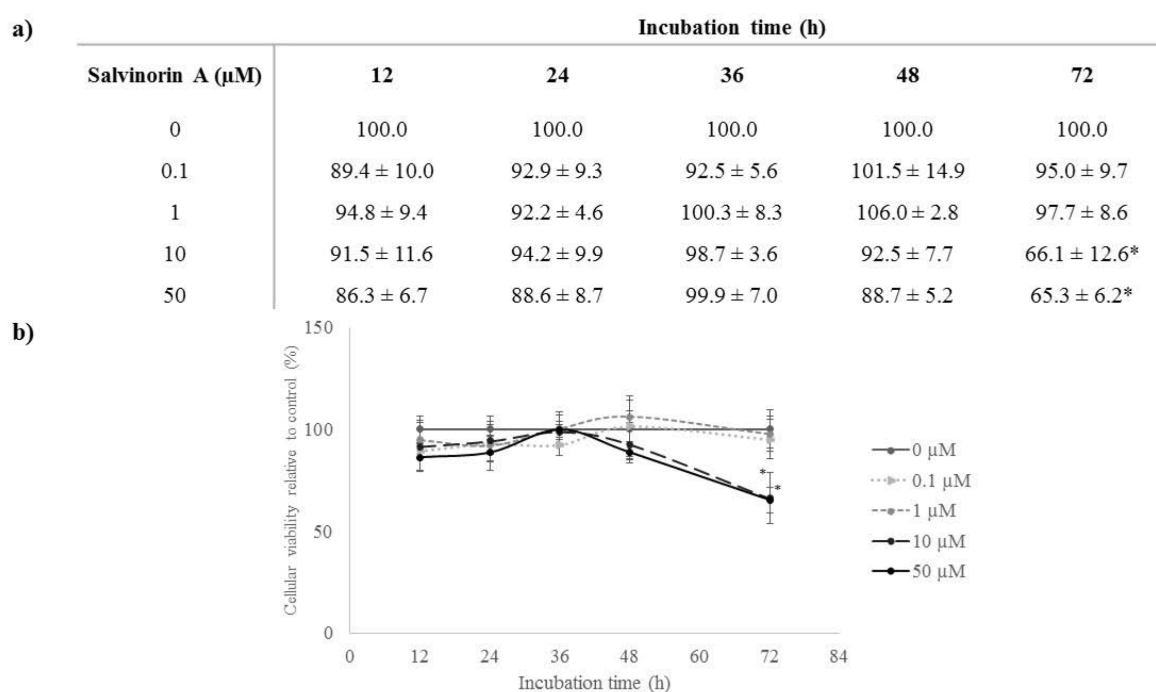
The results showed that the incubation of HepG2 cells with 0.1 and 1 μM SA promote no cytotoxicity while the incubation with both 10 and 50 μM of SA are cytotoxic to these cells as their cellular viability decreased comparing to control.

Regarding the WRL-68 cells, as showed in figure 2, the incubation with0.1, 1 and 10 μM of SA did not decreased the cellular viability of these cells. Otherwise, data showed that 50 μM are cytotoxic for WRL-68 cells for incubations longer than 12h as the CV decreased to approximately one half (24 h: 69.46 ± 6.03; 36 h: 63.72 ± 5.48; 48 h: 50.01 ± 4.46 and 72h: 49.71 ± 2.14).

Evaluation of SA effect on gene expression

Real-time PCR assays were performed in HepG2 and WRL68 to evaluate the mRNA expression of ApoB100,

Figure 1: Cellular viability of HepG2 cell line after exposure to 0 (control), 0.1, 1, 10 and 50 μM of SA for 12, 24, 36, 48 and 72h: a) Data is expressed as the mean ± standard error and differences between treated and control groups were determined by Student´s t test and considered significant when $p < 0.05$(*); b) Graphical representation of data.



a)

	Incubation time (h)				
Salvinorin A (μM)	12	24	36	48	72
0	100.0	100.0	100.0	100.0	100.0
0.1	89.4 ± 10.0	92.9 ± 9.3	92.5 ± 5.6	101.5 ± 14.9	95.0 ± 9.7
1	94.8 ± 9.4	92.2 ± 4.6	100.3 ± 8.3	106.0 ± 2.8	97.7 ± 8.6
10	91.5 ± 11.6	94.2 ± 9.9	98.7 ± 3.6	92.5 ± 7.7	66.1 ± 12.6*
50	86.3 ± 6.7	88.6 ± 8.7	99.9 ± 7.0	88.7 ± 5.2	65.3 ± 6.2*

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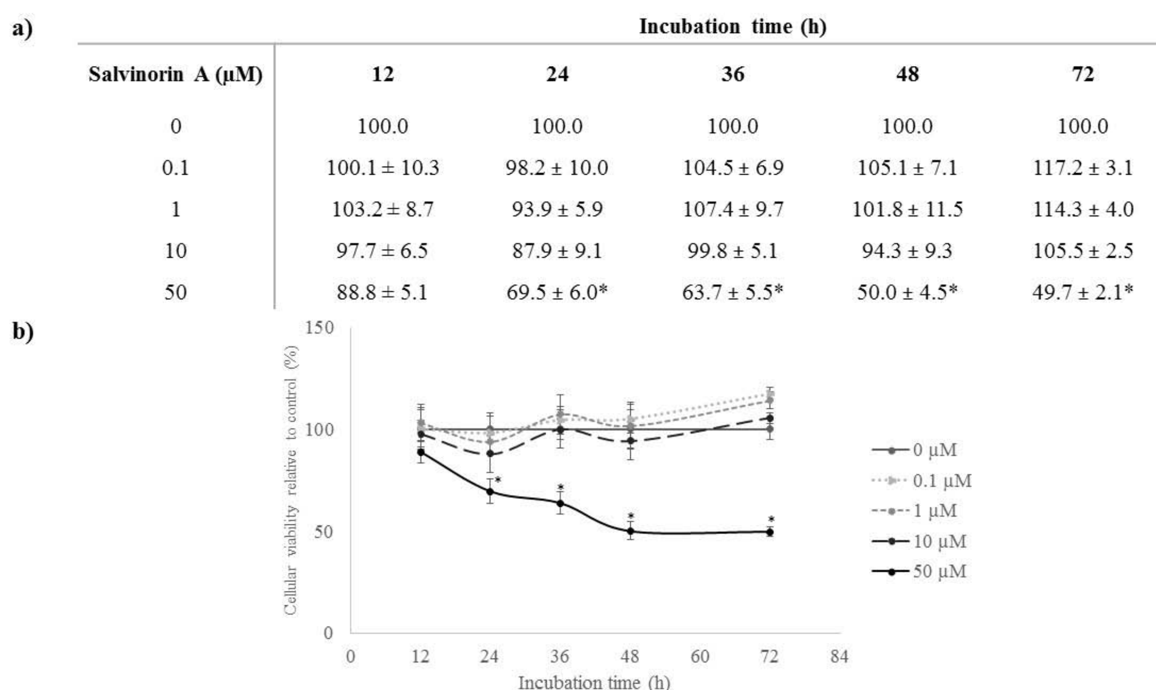
Figure 2: Cellular viability of WRL 68 cell line after exposure to 0 (control), 0.1, 1, 10 and 50 μM of SA for 12, 24, 36, 48 and 72h: a) Data is expressed as the mean ± standard error and differences between treated and control groups were determined by Student´s t test and considered significant when p< 0.05(*); b) Graphical representation of data.



a)

Salvinorin A (μM)	Incubation time (h) 12	24	36	48	72
0	100.0	100.0	100.0	100.0	100.0
0.1	100.1 ± 10.3	98.2 ± 10.0	104.5 ± 6.9	105.1 ± 7.1	117.2 ± 3.1
1	103.2 ± 8.7	93.9 ± 5.9	107.4 ± 9.7	101.8 ± 11.5	114.3 ± 4.0
10	97.7 ± 6.5	87.9 ± 9.1	99.8 ± 5.1	94.3 ± 9.3	105.5 ± 2.5
50	88.8 ± 5.1	69.5 ± 6.0*	63.7 ± 5.5*	50.0 ± 4.5*	49.7 ± 2.1*

Ces1, Cyt C, CYP 1A2, 2D6, 3A4 and UGT 1A1 after incubation during 12, 24 and 72h with 0 and 1 μM SA.

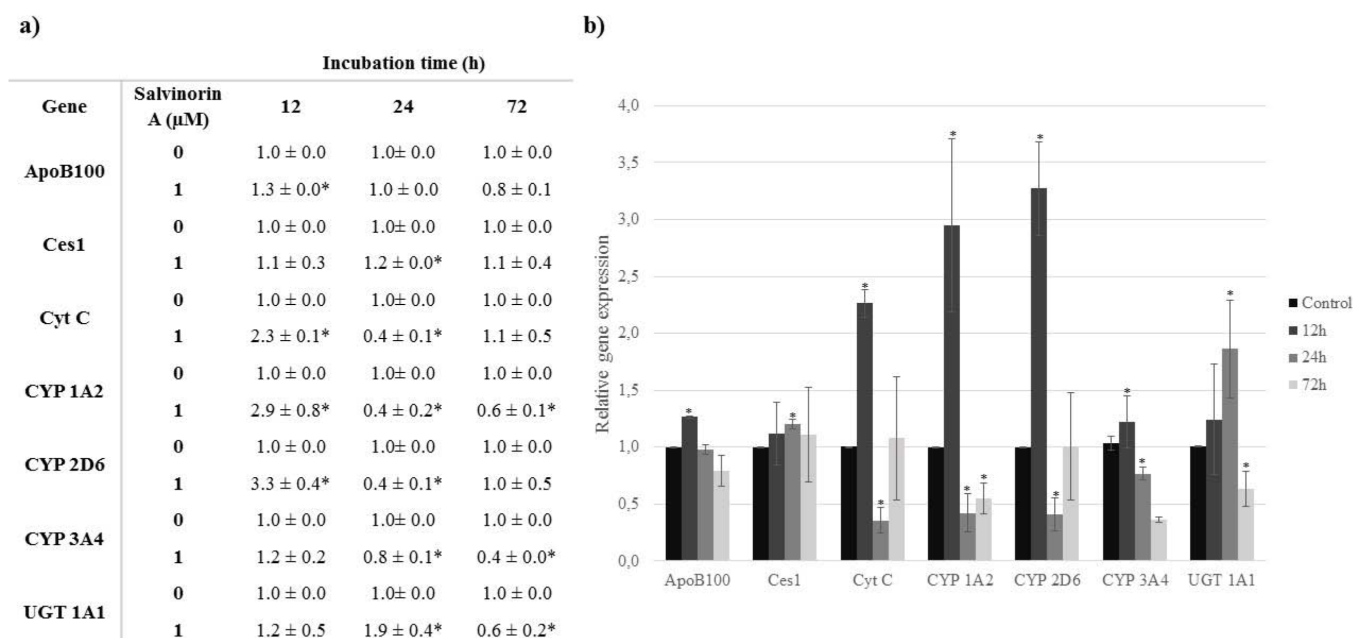
In HepG2 cells, data (Figure 3) showed that the relative mRNA expression of both ApoB100 and Ces1 isslightly increasedafter an incubation with SAfor both 12h (1.3 ± 0.0) and 24h (1.2 ± 0.0).Similarly, the relative Cyt C mRNAexpression after 12h of incubation with SA doubled(2.3 ± 0.1), but decreased after 24h of incubation with the compound (0.4 ± 0.1) and returned to its basal levels after 72h. Also, UGT 1A1 gene expression increased to approximately two times after a 24h SA incubation (1.9 ± 0.4)and decreased after 72h to approximately one half (0.6 ± 0.2).

Regarding mRNA CYP450 expression, results showed that after 12h of incubation with SA there is an increase of the relative mRNA expression of CYP 1A2 (2.9 ± 0.8) and CYP 2D6 (3.3 ± 0.4). After 24h of incubation with the compound, the relative mRNA expression of all CYP450 isoforms decreased dramatically to approximately one half and, after 72 h of incubation, the relative mRNA expression remained decreased, except for CYP 2D6, that returned to basal mRNA expression levels.

As showed in Figure (4), in WRL68 cells the relative mRNA expression of ApoB100 and CYP 3A4 increased after 12h of incubation with SA (1.5 ± 0.4 and 2.2 ± 0.3, respectively) and returned to basal levels after a 24- or 72 h-incubation. In relation to Cyt C mRNA expression results showed that with the exception of 24h, which there was observed no alterations, the incubation of WRL 68 with SA promoted an increase in its relative expression both after 12h (0.6 ± 0.0) and 72h (0.5 ± 0.0*).

Also, data showed that CYP 1A2 mRNA expression was

Figure 3: Relative mRNA expression of ApoB100, Ces1, Cit. C, CYP 1A2, CYP 2D6, CYP 3A4 and UGT 1A1 in HepG2 cell line after exposure to 0 (control) and 1 µM of SA for 12, 24 and 72 h:a) Data is expressed as the mean ± standard error and differences between treated and control groups were determined by Student´s t test and considered significant when p< 0.05(*); b) Graphical representation of data.



a)

Gene	Salvinorin A (µM)	Incubation time (h) 12	24	72
ApoB100	0	1.0 ± 0.0	1.0± 0.0	1.0 ± 0.0
	1	1.3 ± 0.0*	1.0 ± 0.0	0.8 ± 0.1
Ces1	0	1.0 ± 0.0	1.0± 0.0	1.0 ± 0.0
	1	1.1 ± 0.3	1.2 ± 0.0*	1.1 ± 0.4
Cyt C	0	1.0 ± 0.0	1.0± 0.0	1.0 ± 0.0
	1	2.3 ± 0.1*	0.4 ± 0.1*	1.1 ± 0.5
CYP 1A2	0	1.0 ± 0.0	1.0± 0.0	1.0 ± 0.0
	1	2.9 ± 0.8*	0.4 ± 0.2*	0.6 ± 0.1*
CYP 2D6	0	1.0 ± 0.0	1.0± 0.0	1.0 ± 0.0
	1	3.3 ± 0.4*	0.4 ± 0.1*	1.0 ± 0.5
CYP 3A4	0	1.0 ± 0.0	1.0± 0.0	1.0 ± 0.0
	1	1.2 ± 0.2	0.8 ± 0.1*	0.4 ± 0.0*
UGT 1A1	0	1.0 ± 0.0	1.0± 0.0	1.0 ± 0.0
	1	1.2 ± 0.5	1.9 ± 0.4*	0.6 ± 0.2*

increased after 24h (1.2 ± 0.0) and 72h (1.8 ± 0.0) of incubation with SA. Otherwise, CYP 2D6 mRNA expression was not altered at any time point studied. Regarding the UGT 1A1, it was observed that its mRNA expression was increased almost 4X (3.7 ± 1.1) after 12h of incubation with SA and dramatically decreased after 24h (0.6 ± 0.2) and 72h (0.1 ± 0.0*). For Ces1 gene there were no data obtained as it was observed no Ces 1 mRNA expression in these cells.

Discussion

Martin ho et al. (2015) evaluated the SA in vitro toxicity in various cell lines and observed that it is dose-, cell- and time- depend [40]. Similarly, here we observed a similar profile as it was showed that 50 µM of SA is cytotoxic to both cell lines and 10 µM of the compound induced also a significant decrease in the cellular viability of cells, in particular, for longer periods of incubation.

Overall, MTT assays showed that 50 µM of SA is cytotoxic at any period of time and for both cell lines and 10 µM promoted a decrease in the CV of HepG2, indicating that, in general, HepG2 are more sensitive to SA than WRL 68 cells.

A previous study reported that 200 to 500 µg of SA are enough to produce intense hallucinogenic effects [7]. Similarly, considering the percentage of SA present in the plant [3], it was observed that 1 µM corresponds to 432 µg of SA and 0,216 g of S. divinorum leaves, thus highlighting the low cytotoxic effects of SA on organism.

ApoB100 encodes for an essential protein involved

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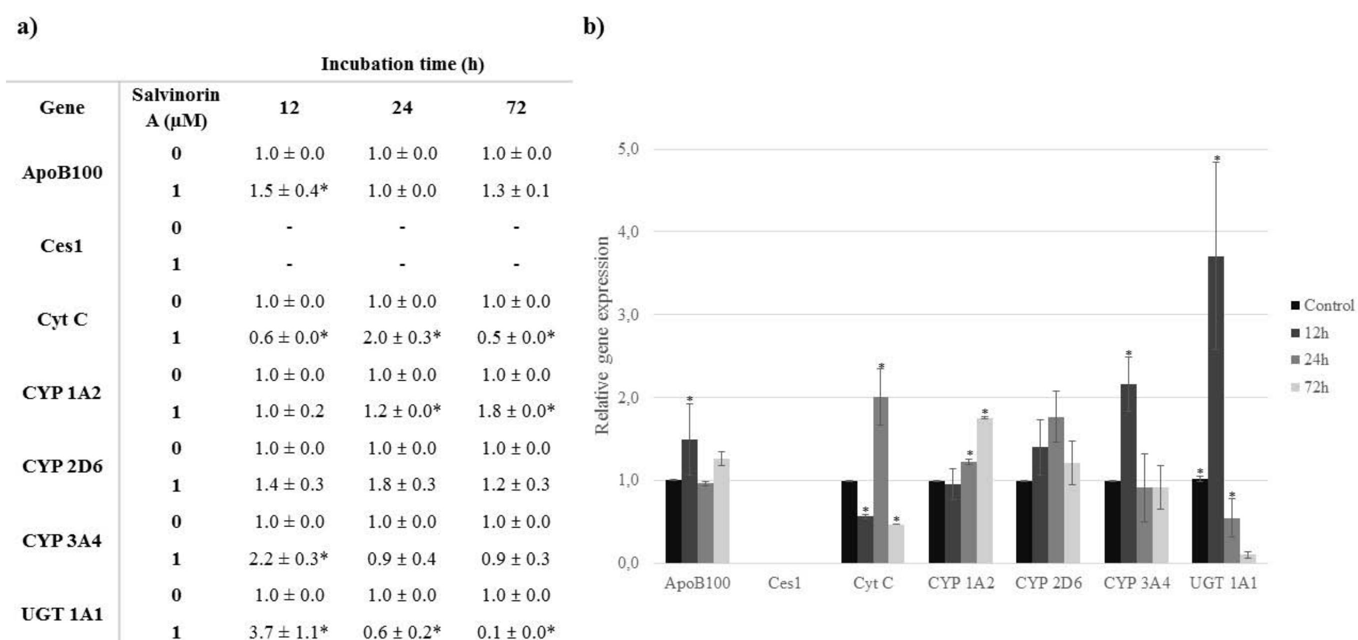
Figure 4: Relative mRNA expression of ApoB100, Ces1, Cit. C, CYP 1A2, CYP 2D6, CYP 3A4 and UGT 1A1 in WRL68 cell line after exposure to 0 (control) and 1 µM of SA for 12, 24 and 72 h:a) Data is expressed as the mean ± standard error and differences between treated and control groups were determined by Student´s t test and considered significant when p< 0.05(*); b) Graphical representation of data.



a)

Gene	Salvinorin A (µM)	Incubation time (h) 12	24	72
ApoB100	0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
	1	1.5 ± 0.4*	1.0 ± 0.0	1.3 ± 0.1
Ces1	0	-	-	-
	1	-	-	-
Cyt C	0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
	1	0.6 ± 0.0*	2.0 ± 0.3*	0.5 ± 0.0*
CYP 1A2	0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
	1	1.0 ± 0.2	1.2 ± 0.0*	1.8 ± 0.0*
CYP 2D6	0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
	1	1.4 ± 0.3	1.8 ± 0.3	1.2 ± 0.3
CYP 3A4	0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
	1	2.2 ± 0.3*	0.9 ± 0.4	0.9 ± 0.3
UGT 1A1	0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
	1	3.7 ± 1.1*	0.6 ± 0.2*	0.1 ± 0.0*

in lipid metabolism, in the secretion and formation of LDL, and a decrease in its expression is linked to some pathologies, ashypercholesterolemia [30]. According to our results, SA was showed to increase ApoB100 mRNA expression, in a cell- and time-dependent manner, which could promote an increase in LDL levels and a decrease of HDL concentration in plasma [43]. However more studies are needed to fully understand SA implication on lipid metabolism as in red wine polyphenolics substances increase LDL receptor expression and suppress ApoB100 secretion lowering cholesterol levels in HepG2 [44].

Ces1 is a very important enzyme in metabolism of xenobiotic and other endogenous compounds that could be harmful to organism [32]. After HepG2 cells 1exposure to SA, the relative mRNA expression of Ces1 slightly increased, which could have an impact in the metabolism of toxic, thus accelerating it. In WRL68 cells, this enzyme was found to be no expression. At this point, our results highlight the importance of the selection of the cell line to perform in vitro studies indicating that, depending on the gene to study, cells could be a suitable, or not, biological model.

Regarding the Cyt C, it is a protein involved in cellular respiration and it playsa main role in apoptosis. In fact, cellular respiration is very important to cell survival and Cyt C is pivotal to this process[45]. In the case of WRL68 cells, it was observed a significant decrease in the Cyt C mRNAexpressionafter incubation with 1 µM of SA for 12 h, which was counteracted after 24h to approximately 2x comparing to controland,after 72h, it drastically

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decreased again to approximately one half. In HepG2 cells, in general, the same profile wasobserved but it was increasedafter 12h of incubation with SA and decreased after 24h returning to basal levels at 72h. Considering the relevance of this protein in organism, it would be considered that SA may lead to a higher or shorter cell, time-dependent, thus causing an impact in apoptosis which, depending on cell type, could be beneficor harmful.

Most CYP450enzyme isoforms are involved in drug metabolism and their bioavailabilityis very important in the degradation and elimination of potential dangerous compounds to organisms [35]. In fact it is remarkable that the metabolism rate of substances are reduced in liver-like diseases because CYP450 enzymes are altered [46]. In general, for shorter periods of exposure to SA it seems that the CYP450enzymes included in the study were all increased, which possiblymay an impact in the increasing of drug metabolism. After 24h of incubation with1 μM of SA,in HepG2 cells these increases were abrogated or decreased while in WRL 68 cells it is increased (CYP 1A2) or without changes (CYP 2D6 and CYP 3A4).

Finally, UGT 1A1 is an enzyme of phase II reactions involved in the detoxification process in organisms, acting in the clearance of compounds that cannot be eliminated after the phase I reactions [38]. In WRL 68 and HepG2 it was observed an increase in its expression after 12 and 24h respectively, followed by a drastic decrease. UGT 1A1 is the only enzyme able to metabolize bilirubin and changes in its expression could lead to jaundice (hyperbilirubinemia) that, if uncontrolled, could reach the brain and cause serious neurologic damage [47]. According to our results, SA consumption, in general, seems to decrease UGT 1A1 expression which could potentiate harmfulbrain consequences.

Overall, the mRNA expression of the genes included in the study increased for shorter periods of exposure to SA and decreased for longer ones, suggesting a putative negative feedback exerted by SA exposure.

Considering the potential therapeutic relevance of S. divinorum and its main constituent, SA, and hepatic genes' expression, overall, the results presented on this studyupdate the current knowledgeon SA actions related to in vitro toxicity and open new perspectives to further studies regardingthe research with S. divinorum and SA.

Conclusion

S. divinorum and its main bioactive compound, SA, present unique properties. In this study, we showed for the first time, that SA has some biological effects in hepatic cells. The in vitro toxicity profile of this plant seems to be relatively safe as the cytotoxic concentrations are higher comparing with the typical consumption range levels. Here we showed that SA could promote both negative and positive effects in organism, in particular in the liver, as it regulates the expression of some genes in hepatic cells, involved in metabolism.

Overall, depending on the cell line, concentration and time of incubation, SA can exert distinct effects both in cytotoxicity and in genes mRNA expression. The results highlighted that at least the continuous and high-dose usage of SA should be carefully monitored, especially in combination with ApoB100, Ces1, Cyt C, CYP 1A2, 2D6, 3A4 and UGT 1A1 target compounds.

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