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Cytotoxic Effects of Salvinorin A, A Major Constituent of *Salvia divinorum*

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Abstract: *S. divinorum* is a psychoactive plant that has been consumed as a recreational drug of abuse in the last years. Salvinorin A is its main constituent, and is responsible for the observed psychoactive effects. Both *S. divinorum* and salvinorin A have become controlled drugs in several countries, but they are not listed in the Schedules of the United Nations Drug Conventions.

Regarding the effects of *S. divinorum* consumption, almost all studies are based on *in vivo* or on surveys, and there are no studies *in vitro* on its toxicity. Furthermore, all studies are focused on the acute toxicological effects of the plant. So, it is of utmost importance to further investigate the effects of *S. divinorum* and salvinorin A, particularly using *in vitro* models, after prolonged exposures. In this context, the present work evaluated the *in vitro* toxicity induced by *S. divinorum* or salvinorin A in six cell lines, through MTT assays and LC_{50} determination.

Overall, results showed that both *S. divinorum* and salvinorin A are cytotoxic, dose- and time-dependent. Also, Hep G2 and Caco 2 (to a lesser extent) cells showed lower sensitivity to *S. divinorum* and salvinorin A when compared to the other studied cell lines.

To our knowledge, this is the first work focused on the *in vitro* toxicity of *S. divinorum* and salvinorin A using a variety of cell lines, which are extensively described in literature and have been widely used in several *in vitro* studies.

Keywords: Cytotoxicity, Salvinorin A, *Salvia divinorum*, LC_{50}, *in vitro*, drug of abuse.

INTRODUCTION

The plant *Salvia divinorum* (also known as "diviner's sage", "magic mint", "mystic sage", "ska Maria" and "ska Pastora") is a perennial member of the *Lamiaceae* (mint) family, indigenous to Sierra of Oaxaca, Mexico [1-3]. It is a psychoactive herb used by Mazatecs to treat a number of medical conditions, including headaches, rheumatism, abdominal swelling and diarrhea, divination and shamanism [2, 4, 5]. In the last few years, *S. divinorum* has also been used as a drug of abuse, particularly by young people in Europe and the USA, due to its potent hallucinogenic effects [3, 5-9]. This herb is easily available both online and in the widespread smartshops, as its distribution is not controlled in most countries [10-13]; for this reason its consumption has rapidly increased, particularly in adolescents and young adults. Neither *S. divinorum* nor salvinorin A are listed in any of the Schedules of the United Nations Drug Conventions. However, in the last years, they have become controlled under drugs legislation in various countries [11].

S. divinorum is often compared to cannabis due to similar effects, which include perceptions of bright light, vivid shapes and colors, object distortions, uncontrolled laughter, dysphoria, overlapping realities, hallucinations, dizziness, uncoordination and, often, incoherent speech, depersonalization and unconsciousness [5, 11, 14]. In fact, in the two last years, several European hospitals have notified the hospitalization of intoxicated patients with *S. Divinorum*, and for this reason the European Union was aware of the abusive consumption by young people.

The part of the plant that is more often consumed is the leaves and to a lesser extent the stems. Usually, it is consumed by inhalation *via* smoking the dried leaves, or *via* volatilization. Moreover, buccal absorption by mastication [1, 7, 15, 16] and, ingesting a tincture or drinking the beverage obtained from the crushing of the leaves is also observed [6]. Depending on the consumption method, pharmacodynamics of *S. divinorum* is relatively rapid: 30 s for smoking and 5 to 10 min for buccal absorption [1, 17]; in humans, inhaled doses of 200 – 500 μg produce profound hallucinations which last for 1 to 2 h [18-20].

It was reported that effects of this herb are mechanistic and structurally distinct from other natural and synthetic hallucinogens, including N,N-dimethyltryptamine, 4-bromo-2,5-dimethoxyphenylisopropylamine, psilocybin and mescaline, lysergic acid diethylamide (LSD), and ketamine [8, 15, 21]. Although the psychotropic effects of *S. divinorum* have been widely investigated, the chemical compound(s) responsible for these properties have not been extensively studied yet.

The psychoactive component of *S. divinorum* is the structurally unique furanolactone neoclerodane diterpene Salvi-

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norin A (SA) [1, 17, 22]. *S. divinorum* also contains other structurally related compounds, which are present in the plant at lower concentrations than SA – named salvinorins B to I, salvicinins A and B, divinatorins A to E and salvidivins A to D [23] – but their potential biological activity, still remains unknown [3, 20]. A previous study showed that, contrarily to SA, salvinorin B (a major metabolite of SA) was inactive at kappa-opioid receptor (KOR) [24, 25].

KOR is one of the receptors that bind opioid-type compounds in the brain and is involved in pain perception, mood and motor control [26]. It is mainly distributed in the central nervous system but also in several peripheral tissues including the lung, small and large intestines, adrenals, kidney, stomach, spleen, heart, testis, ovary and uterus [27]. It is an important therapeutic target for the treatment of several psychiatric and non-psychiatric disorders [5, 8], including analgesia, schizophrenia, Alzheimer's disease, stimulant dependence and other mood disorders [28, 29].

The discovery of KOR, as the molecular target of SA, both *in vitro* and *in vivo*, suggested that its activation promoted the hallucinogenic effects produced by SA, thus highlighting the importance of this molecule in drug development and discovery. However, SA has a distinct profile of activation of KOR comparing to other hallucinogens such as LSD [6], as it does not activate the serotonin 2A (5-HT_{2A}) receptor [8], the major site of activity of classical hallucinogens [8, 20, 30].

SA is primarily found in the leaves of the plant and it is the first non-alkaloid hallucinogen and the first identified potent non-nitrogenous selective KOR agonist [5, 8, 31-34]. SA can be either absorbed through the buccal membrane, requiring a minimum concentration to be active [35], or absorbed *via* inhalation of the vaporized compound, leading to a rapid onset of the psychoactive effects. However, SA is rapidly degraded in the gastrointestinal tract, and thus only a small amount of the compound is absorbed orally. The duration of the effects is short, as SA is mainly deactivated in the gastrointestinal tract [1, 36]. The distribution of SA is rapid and it is metabolized in the liver and gallbladder [37, 38].

As already stated, SA was identified as the major bioactive constituent of *S. divinorum* and is responsible for the psychoactive actions of this plant. Nevertheless, to our knowledge, the few existing studies on the matter are focused only on *in vivo* or on surveys, and there are no studies which further explore *in vitro* toxicity of SA and *S. divinorum*. Also, considering the availability, use, controversy and little information regarding the effects of prolonged exposure, it is of utmost importance to further investigate the chronic effects of *S. divinorum* and SA on the organism, providing information related to this novel drug of abuse. In this context, the present work intents to evaluate the S. divinorum extract- or SA-induced *in vitro* toxicity on several cell lines from dopaminergic neurons, lung, liver, kidney and intestine (N 27, A 549, Caco 2, Hep G2, Hek 293, COS 7).

MATERIAL AND METHODS

Plant Material and Chemicals

Twenty grams of dried leaves of *S. divinorum* were macerated with 25 mL 10 % dimethyl sulfoxide (DMSO; Sigma-Aldrich, Inc., St. Louis, MO, USA) and 0.1 % 0.1 M HCl (Merck Millipore, Billerica, MA, USA). The solution was heated for approximately 3 h at 45 ºC, filtered and the resulting supernatant was stored at 4 ºC. Before the experiments, the concentration of SA present in the extract was determined by gas chromatography-tandem mass spectrometry (GC-MS/MS). For chromatographic analysis, an HP 7890A gas chromatography system coupled to a 7000B triple quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany), and a MPS2 autosampler from Gerstel (Mülheim an der Ruhr, Germany) was used. For the separation of the analytes a capillary column (30-m x 0.25-mm I.D., 0.25-µm film thickness) with 5 % phenylmethylsiloxane (HP-5 MS) from J & W Scientific, Folsom, CA, USA) was used.

A stock solution of SA (LGC Standards, Barcelona, Spain) at 0.001 M was prepared by dissolving the standard in 10 % DMSO (Sigma-Aldrich, Inc.) and 0.1 % 0.1 M HCl (Merck Millipore), and was stored at 4 ºC.

Determination of SA in *S. divinorum* Plant

SA was quantified in the plant by gas chromatography-tandem mass spectrometry. Briefly, 4 g of dried leaves of *S. divinorum* were macerated with 5 mL of methanol (Merck Millipore, Billerica, MA, USA). The extracts were evaporated to dryness, and were dissolved in 50 µL of methanol, transferred to autosampler vials and 2 µL were injected into the GC-MS/MS. The conditions of gas chromatography and mass spectrometry are described elsewhere [11]. The quantity of SA present in these leaves was 0.05 mg/g (Fig. **1**). The study was continued using 20 g of *S. divinorum* in 25 mL of solution and the equivalent of SA standard (0.001 M).

Cell Lines and Culture

The cell lines were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured at 37 ºC in a humidified 5 % CO_2 incubator in 75 or 175 cm^2 culture flasks.

The six cell lines included in the study were: N 27 cells - rat mesencephalic dopaminergic neurons; A 549 - human adenocarcinoma alveolar basal / lung cells; Caco 2 - heterogeneous human colorectal adenocarcinoma cells; Hep G2 - human hepatocellular carcinoma cells; and, two cell lines derived from kidney cells - COS 7 - Fibroblast-like derived from monkey, and Hek 293 - human embryonic 293 cells, which cover most of the tissues or organs that, theoretically, are more susceptible to this drug of abuse. In fact, SA rapidly crosses the blood-brain barrier [40], thus penetrating in the central nervous system. Therefore, N 27 cells were included because these cells possess all the physiological and biochemical properties of dopaminergic neurons [41] and are usually used as an *in vitro* model for neuronal diseases, particularly Parkinson's disease [42]. Furthermore, SA is consumed by inhalation, *via* smoking the dried leaves of *S. divinorum* or *via* volatilization [1]. So, this study included the A 549 cell line, a type II pulmonary epithelium often used as a cell model for drug metabolism [43] and alveolar toxicity. SA may also be consumed orally, but through it is readily degraded in the gastrointestinal tract [1, 36]. Besides, Caco 2 cells were also included, as they are widely used as intestinal

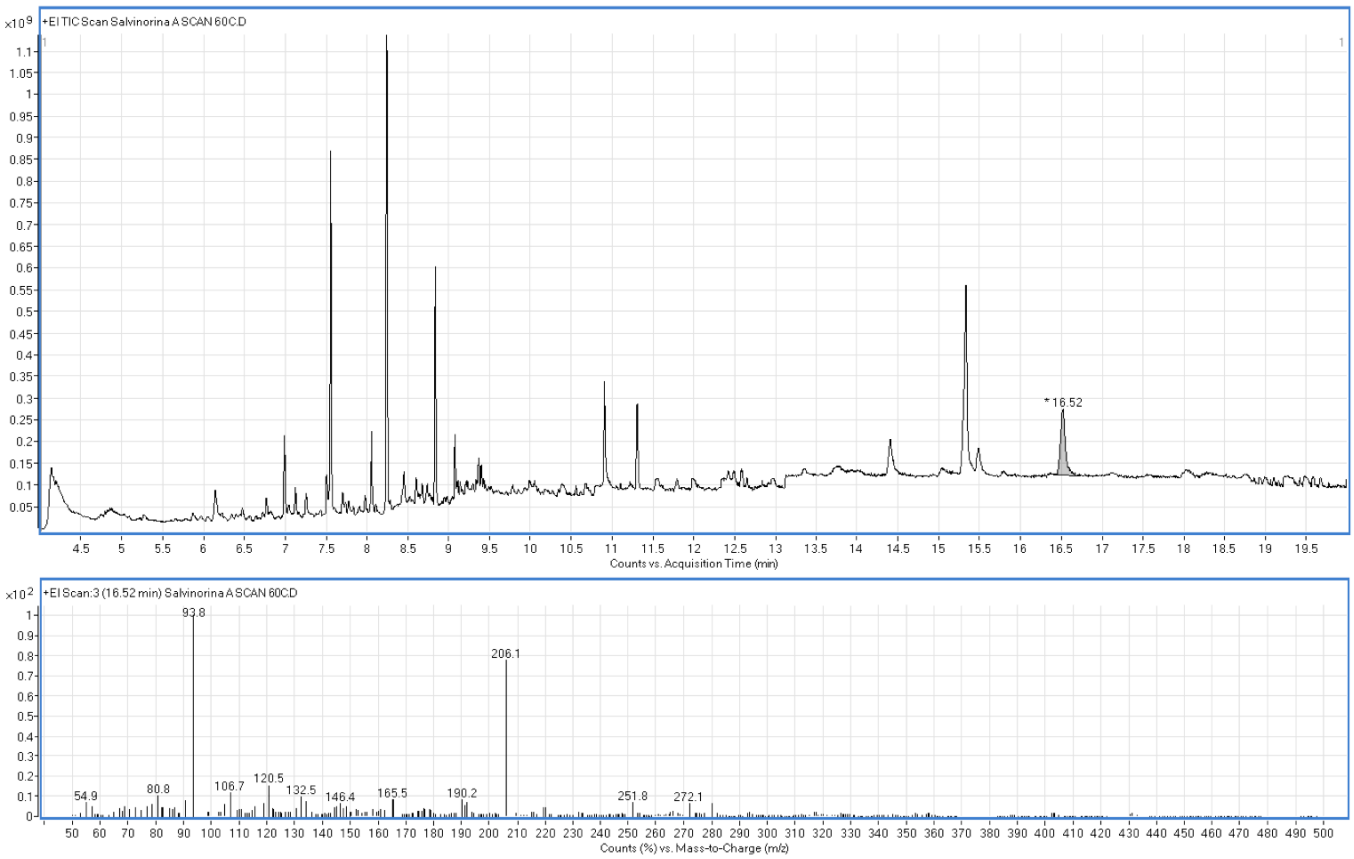

Fig. (1). Chromatogram obtained by injection of a processed leaves sample into GC/M/MS and respective mass spectrum (scan mode). The retention time of Salvinorin A was 16.52 min.

barrier model [44, 45] and in a series of *in vitro* transport studies. Additionally, as SA has a rapid distribution and it is metabolized by UGT2B7, CYP2D6, CYP1A1, CYP2E1 and CYP2C18 [15] in the liver and gallbladder [37,38], we have also evaluated the cytotoxicity of both compounds in Hep G2 cells, one of the most used *in vitro* models in pharmacological, toxicological and drug metabolism studies [46,47]. Finally, to ascertain if *S. divinorum* and SA induced toxicity on kidney-derived cells, MTT assays were also performed on COS 7 and Hek 293 cells because both cell lines are vastly used in molecular biology and *in vitro* studies. The main difference between COS 7 and Hek 293 cells is that the first was established from a CV-1 cell line derived from kidney cells of the African green monkey (Cercopithecus aethiops) and the second derived from human embryonic kidney cells [48, 49].

N 27 and Caco 2 cells were maintained in RPMI 1640 medium (Sigma-Aldrich, Inc.) supplemented with 10 % fetal bovine serum (FBS; Sigma-Aldrich, Inc.), 2 mM L-glutamine (Sigma-Aldrich, Inc.), 0.1 mM MEM non-essential amino acids (NEAA; Sigma-Aldrich, Inc.), 100 U/mL penicillin (Sigma-Aldrich, Inc.) and 100 µg/mL streptomycin (Sigma-Aldrich, Inc.). Hek 293 cells were cultured in EMEM (Sigma-Aldrich, Inc.) supplemented with 10 % FBS, 2 mM NEAA, 100 U/mL penicillin and 100 µg/mL streptomycin. Hep G2, A 549 and COS 7 cells were maintained in DMEM high glucose medium (Sigma-Aldrich, Inc.) supplemented with 10 % FBS, 100 U/mL penicillin and 100 µg/mL streptomycin.

Cell Treatments

When cells were approximately 90 - 95 % confluent, they were tripsinized and seeded in quadruplicate in 96 - well culture plates (10 000 cells *per* well; Nunc, Apogent, Denmark), and were left to adhere for 24 - 48 h. Then, after reaching 80 - 90 % confluence in the multiwell plates, the medium was replaced for serum-free correspondent medium, and after 12 h cells were incubated with 0, 0.1, 1, 10 or 50 µM of SA or *S. divinorum* extract for 12, 24, 48 or 60 h, in the appropriate serum-free medium. The DMSO carry-over from the extract or the SA stock solutions was kept below 0.1 % to prevent solvent-induced cytotoxicity . Each experiment was repeated at least three times, independently.

Cellular Viability Assay

In vitro cytotoxicity of cells was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma- Aldrich, Inc.) assay, as described by Martinho *et al.* (2012) [50]. Briefly, replicates of the experiments were incubated with 0.5 mg/mL of MTT prepared in Krebs solution (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 1 mM $CaCl_2$, 6 mM glucose and 10 mM hepes, pH 7.4) for 120 - 180 min at 37°C. MTT is converted by viable cells into water-insoluble precipitates which solubilize after the addition of 40 mM HCl in isopropanol. After incubating for 5 min in the dark with gentle agitation, the absorbance of each replicate was measured at 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). Each experiment was repeated three times, independently.

a)

Salvia divinorum

% of viable cells relative to controls — Incubation (h)	0 µM	0.1 µM	1 µM	10 µM	50 µM
12	100.0	89.7 ± 4.4*	98.0 ± 1.7	89.6 ± 4.5*	64.6 ± 5.7*
24	100.0	80.2 ± 3.9*	88.9 ± 1.7*	73.7 ± 2.9*	27.7 ± 0.8*
48	100.0	113.2 ± 5.2*	107.5 ± 0.7*	103.0 ± 9.4	41.1 ± 3.9*
60	100.0	98.1 ± 8.1	78.6 ±4.5*	61.4 ± 3.5*	42.8 ± 1.9*

b)

Salvinorin A

0 µM	0.1 µM	1 µM	10 µM	50 µM
100.0	89.9 ± 5.6*	90.7 ± 4.8*	76.9 ± 0.6*	63.4 ± 6.4*
100.0	85.7 ± 6.4*	88.1 ± 4.1*	73.0 ± 5.7*	33.6 ± 2.6*
100.0	99.3 ± 7.8	96.8 ± 4.9	106.8 ± 5.9	48.6 ± 5.4*
100.0	99.1 ± 4.2	102.3 ± 6.3	92.1 ± 6.6	48.3 ± 0.6*

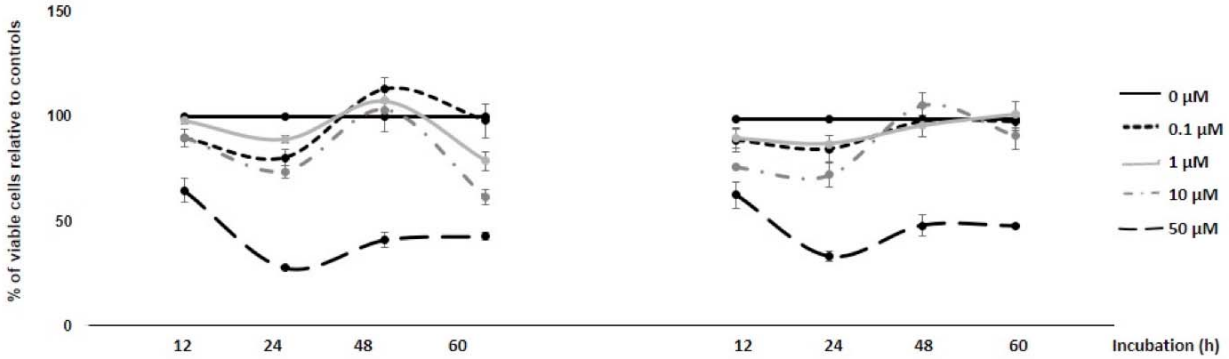

Fig. (2). Cellular viability of N 27 cells after exposure to 0 (control), 0.1, 1, 10 or 50 µM of *S. divinorum* (**a**) or SA (**b**) for 12, 24, 48 or 60 h: Percentage of viable cells relative to controls ± standard deviation (* $p < 0.05$). Data resulted from three independent assays.

Statistical Analysis

The percentage of cell viability relative to controls (CV) was calculated using the formula:

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

Data obtained with the CV calculation were compared by means of one-way ANOVA followed by Student's *t* tests. All results are expressed as mean ± standard deviation and were considered statistically significant whenever $p < 0.05$.

Sigmoid dose-response curves for cell lines were plotted to calculate the concentration, in each incubation period, that kills 50 % of cells (LC_{50}): data were fit to a one-site model with a non-linear regression analysis using GraphPad Prism software (version 5) (GraphPad® Software Inc., La Jolla, CA, USA).

RESULTS

Evaluation of the Cytotoxic Activity of *S. divinorum* and *SA*

As presented in Fig. (**2**), for any tested period of time, 50 µM of both *S. divinorum* extract and SA induced significant toxicity on N 27 cells. Concerning the remaining concentrations (0.1, 1 and 10 µM), the cytotoxic effect of the extract is more evident than that of SA, particularly for periods of incubation higher than 24 h. The incubation for 12 and 24 h with 0.1, 1 and 10 µM of *S. divinorum* extract and SA slightly decreased cellular viability of these cells, and after 48 h it increased to values close to the controls. However, after 60 h of incubation with *S. divinorum* extract, the viability of cells decreased again.

Data in Fig. (**3**) show that after 12 h of incubation, only 50 µM of *S. divinorum* extract or SA decreased cellular viability of A549 cells to 73.1 ± 6.6 and 67.0 ± 4.2, respectively. Furthermore, 24 and 48 h of incubation with *S. divinorum* extract or SA were cytotoxic for almost all concentrations, and the only one that did not compromise their viability was 0.1 µM. Moreover, after 60 h all concentrations promoted a decrease in the viability of these cells for both *S. divinorum* and SA, particularly at 50 µM.

Data obtained for Caco 2 cells (Fig. **4**) show that 50 µM of *S. divinorum* extract, or SA, was cytotoxic in all tested time periods. In fact, cellular viability of Caco 2 decreased continuously after 12, 24 and 48 h of incubation, but after 60 h the percentage of viable cells slightly increased to 72.4 ± 7.2 %, when compared to controls. Regarding the remaining studied concentrations, the profile was somewhat more unstable, suggesting the presence of other compounds in the extract that attenuate SA-promoted toxicity.

For Hep G2 cells, results showed that 50 µM of both *S. divinorum* extract and SA promoted a decrease in the cellular viability after 12, 24, 48 and 60 h of incubation and, in the period of 60 h this effect was slightly lower for SA when compared to the extract (74.7 ± 7.3 and 52.0 ± 6.4 %, respectively) (Fig. **5**). Also, both *S. divinorum* extract and SA at 0.1, 1 and 10 µM are only slightly toxic for these cells after 12 and 24 h of incubation, and after 48 or 60 h cellular viability was not compromised or even has increased when compared to controls.

From the results it may also be pointed that 50 µM of *S. divinorum* extract or SA was cytotoxic for both COS 7 and Hek 293 cells, for all included time periods (Figs. **6** and **7**). The viability of these cells incubated with 50 µM of *S. divinorum* extract or SA decreased to values near 10 % in all tested periods, while in COS 7 this lower percentage of viable cells was only observed after 48 or 60 h of incubation. Moreover, on COS 7 cells, both for *S. divinorum* extract and SA, it was observed a decrease in the percentage of cellular viability after 48 h of incubation for all concentrations, and 10 µM was cytotoxic as well after 24 h. Conversely, after 12 h

a)

% of viable cells relative to controls	*Salvia divinorum*				
Incubation (h)	0 µM	0.1 µM	1 µM	10 µM	50 µM
12	100.0	123.0 ± 4.9*	98.5 ± 6.0	90.2 ± 9.5	73.1 ± 6.6*
24	100.0	104.3 ± 8.3	71.4 ± 5.2*	79.6 ± 2.6*	61.8 ± 6.6*
48	100.0	99.3 ± 4.9	75.0 ± 3.2*	59.4 ± 5.5*	36.0 ± 6.8*
60	100.0	87.6 ± 5.4*	80.5 ± 5.2*	63.1 ± 7.9*	26.0 ± 3.3*

b)

	Salvinorin A				
0 µM	0.1 µM	1 µM	10 µM	50 µM
100.0	120.4 ± 6.2*	95.6 ± 3.4	94.5 ± 8.6	67.0 ± 4.2*
100.0	96.3 ± 1.9	79.5 ± 4.2*	76.3 ± 4.3*	54.7 ± 6.4*
100.0	105.1 ± 2.1*	88.0 ± 1.5*	71.0 ± 6.8*	36.6 ± 7.2*
100.0	85.5 ± 8.5*	64.3 ± 1.0*	47.9 ± 0.8*	23.1 ± 2.9*

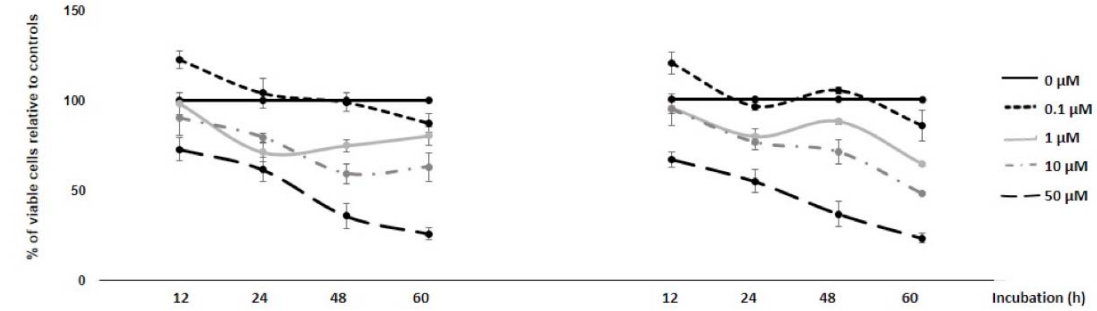

Fig. (3). Cellular viability of A 549 cells after exposure to 0 (control), 0.1, 1, 10 or 50 µM of *S. divinorum* (**a**) or SA (**b**) for 12, 24, 48 or 60 h: Percentage of viable cells relative to controls ± standard deviation (* $p < 0.05$). Data resulted from three independent assays.

a)

% of viable cells relative to controls	*Salvia divinorum*				
Incubation (h)	0 µM	0.1 µM	1 µM	10 µM	50 µM
12	100.0	93.0 ± 4.9	90.0 ± 2.4*	89.7 ± 3.2*	73.2 ± 6.4*
24	100.0	94.8 ± 1.9	103.2 ± 6.7	85.0 ± 6.8*	52.5 ± 8.5*
48	100.0	85.1 ± 6.8*	79.0 ± 4.6*	96.5 ± 7.3	37.5 ± 8.0*
60	100.0	123.8 ± 1.4*	105.9 ± 6.5	82.3 ± 6.2*	66.5 ± 7.3*

b)

	Salvinorin A			
0 µM	0.1 µM	1 µM	10 µM	50 µM
100.0	107.5 ± 2.9*	94.7 ± 7.1	100.0 ± 5.7	74.2 ± 1.9*
100.0	92.9 ± 6.2	121.3 ± 3.7*	105.2 ± 3.9	65.1 ± 1.9*
100.0	78.4 ± 9.0*	54.2 ± 9.4*	42.3 ± 2.9*	42.1 ± 8.3*
100.0	95.9 ± 11.8	112.7 ± 3.9*	115.3 ± 0.7*	72.4 ± 7.2*

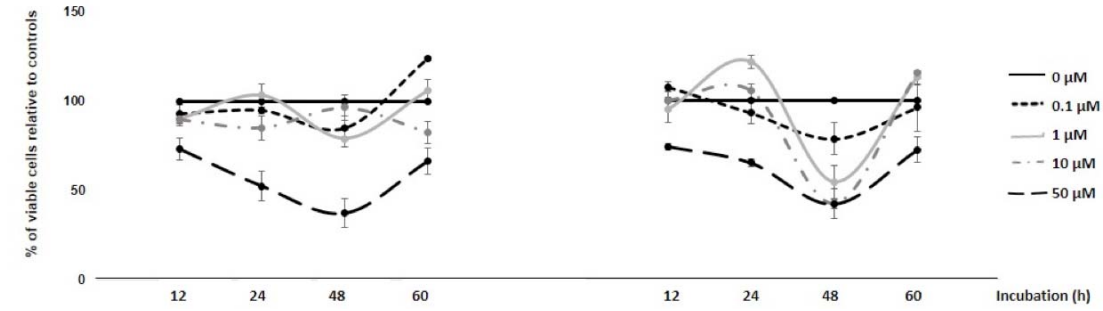

Fig. (4). Cellular viability of Caco 2 cells after exposure to 0 (control), 0.1, 1, 10 or 50 µM of *S. divinorum* (**a**) or SA (**b**) for 12, 24, 48 or 60 h: Percentage of viable cells relative to controls ± standard deviation (* $p < 0.05$). Data resulted from three independent assays.

a)

% of viable cells relative to controls	*Salvia divinorum*				
Incubation (h)	0 µM	0.1 µM	1 µM	10 µM	50 µM
12	100.0	103.3 ± 6.6	95.8 ± 8.7	93.4 ± 3.3*	64.3 ± 3.0*
24	100.0	91.6 ± 6.8*	91.5 ± 3.6*	92.6 ± 4.6*	38.3 ± 6.4*
48	100.0	108.0 ± 7.9*	104.7 ± 1.7	108.6 ± 3.5*	42.8 ± 4.4*
60	100.0	130.8 ± 7.5*	108.8 ± 6.0*	122.9 ± 3.5*	52.0 ± 6.4*

b)

	Salvinorin A				
0 µM	0.1 µM	1 µM	10 µM	50 µM
100.0	91.8 ± 7.5	96.1 ± 1.8	92.9 ± 7.1	61.9 ± 3.8*
100.0	82.4 ± 4.2*	86.9 ± 5.5*	99.5 ± 3.7	37.8 ± 7.3*
100.0	105.8 ± 7.6	98.5 ± 4.9	121.5 ± 4.9*	52.7 ± 5.5*
100.0	125.1 ± 4.3*	113.4 ± 8.3	146.8 ± 3.1*	74.7 ± 7.3*

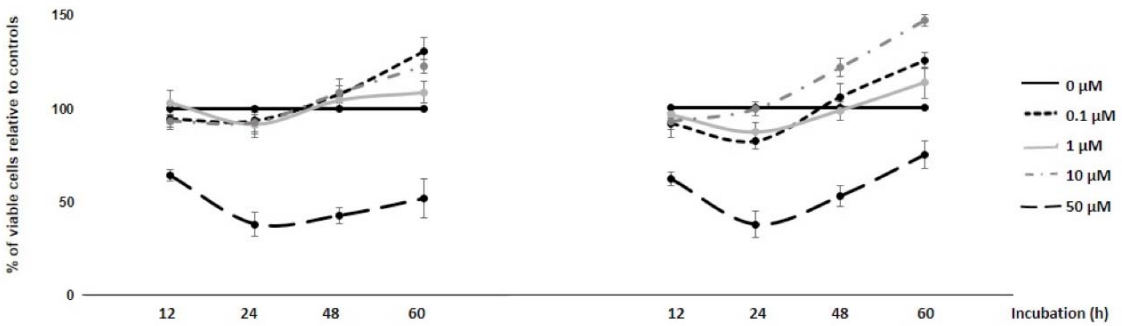

Fig. (5). Cellular viability of Hep G2 cells after exposure to 0 (control), 0.1, 1, 10 or 50 µM of *S. divinorum* (**a**) or SA (**b**) for 12, 24, 48 or 60 h: Percentage of viable cells relative to controls ± standard deviation (* $p < 0.05$). Data resulted from three independent assays.

a)

Salvia divinorum

% of viable cells relative to controls — Incubation (h)	0 µM	0.1 µM	1 µM	10 µM	50 µM
12	100.0	108.3 ± 0.4*	93.3 ± 6.2	83.8 ± 7.3*	45.5 ± 6.9*
24	100.0	125.9 ± 1.3*	107.5 ± 3.6*	78.9 ± 6.4*	38.4 ± 8.9*
48	100.0	87.7 ± 4.4*	92.1 ± 2.4*	62.7 ± 7.4*	13.0 ± 0.6*
60	100.0	75.9 ± 3.4*	73.3 ± 6.7*	52.5 ± 2.9*	8.5 ± 0.4*

b)

Salvinorin A

% of viable cells relative to controls — Incubation (h)	0 µM	0.1 µM	1 µM	10 µM	50 µM
12	100.0	108.1 ± 0.0*	104.0 ± 2.6	117.0 ± 7.2*	51.1 ± 2.8*
24	100.0	105.7 ± 7.5	103.4 ± 0.6	85.2 ± 4.6*	31.9 ± 5.3*
48	100.0	96.6 ± 2.2	79.6 ± 1.0*	51.7 ± 4.0*	11.7 ± 0.3*
60	100.0	98.9 ± 3.3	90.5 ± 5.6*	48.2 ± 0.5*	8.5 ± 0.5*

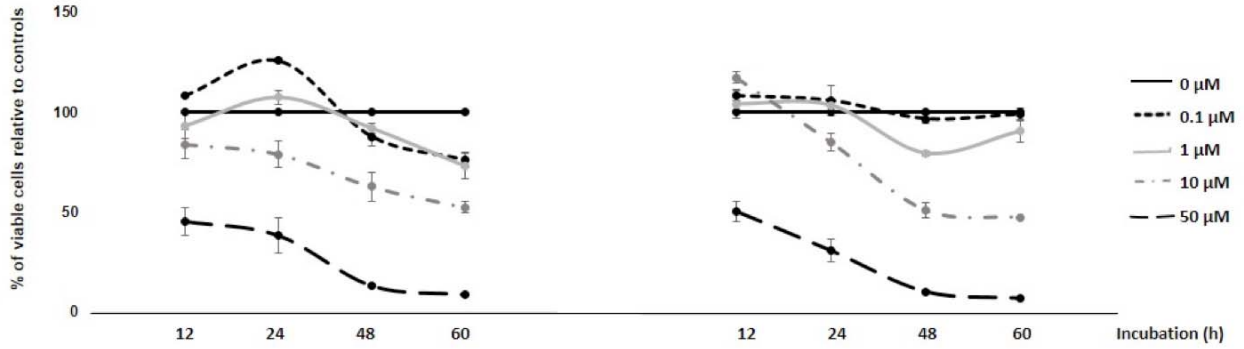

Fig. (6). Cellular viability of COS 7 cells after exposure to 0 (control), 0.1, 1, 10 or 50 µM of *S. divinorum* (**a**) or SA (**b**) for 12, 24, 48 or 60 h: Percentage of viable cells relative to controls ± standard deviation (* $p < 0.05$). Data resulted from three independent assays.

a)

Salvia divinorum

% of viable cells relative to controls — Incubation (h)	0 µM	0.1 µM	1 µM	10 µM	50 µM
12	100.0	116.8 ± 0.2*	95.8 ± 8.7	98.2 ± 2.9	5.2 ± 0.1*
24	100.0	116.0 ± 0.9*	119.5 ± 3.6*	91.0 ± 0.9*	5.7 ± 0.2*
48	100.0	87.9 ± 4.0*	94.4 ± 1.9*	67.5 ± 7.3*	9.1 ± 0.8*
60	100.0	101.1 ± 0.0	103.2 ± 3.6	89.3 ± 2.7*	13.6 ± 2.2*

b)

Salvinorin A

% of viable cells relative to controls — Incubation (h)	0 µM	0.1 µM	1 µM	10 µM	50 µM
12	100.0	120.1 ± 8.1*	107.2 ± 5.3	96.8 ± 3.1	5.3 ± 0.1*
24	100.0	119.4 ± 5.5*	120.4 ± 6.1*	96.7 ± 5.1	5.7 ± 0.2*
48	100.0	100.6 ± 7.1	93.9 ± 4.1	83.9 ± 1.2*	7.6 ± 0.3*
60	100.0	108.4 ± 8.3	103.8 ± 4.2	93.5 ± 6.7	11.3 ± 0.6*

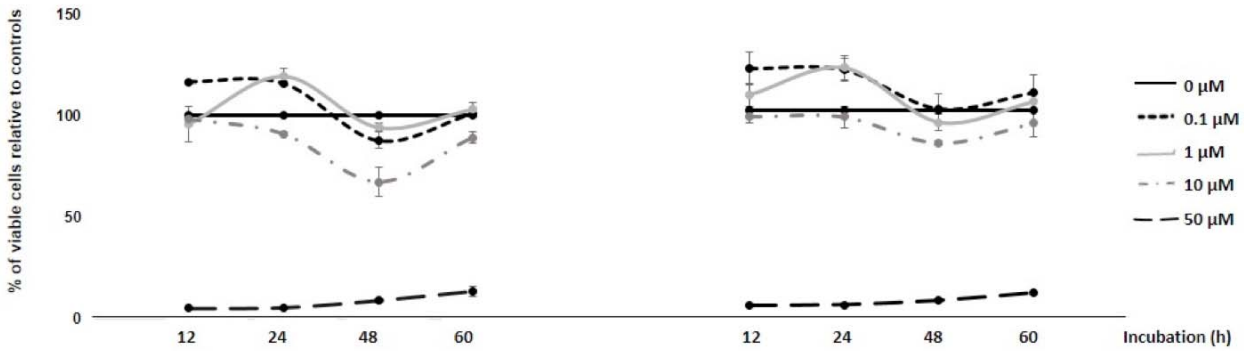

Fig. (7). Cellular viability of Hek 293 cells after exposure to 0 (control), 0.1, 1, 10 or 50 µM of *S. divinorum* (**a**) or SA (**b**) for 12, 24, 48 or 60 h: Percentage of viable cells relative to controls ± standard deviation (* $p < 0.05$). Data resulted from three independent assays.

of incubation no concentration revealed toxicity on COS 7 cells. Regarding Hek 293 cells, their viability was higher than the corresponding COS 7 cells.

DISCUSSION

Considering the data obtained in N 27 cells, it may be pointed out that 50 µM of both *S. divinorum* extract and SA is significantly cytotoxic for all studied periods of time, while for the remaining concentrations (0.1, 1 and 10 µM) the cytotoxic effect of the overall extract is more evident than that of SA, particularly for incubation periods higher than 24 h. Also, after 48 h of incubation with 0.1, 1 and 10 µM of either compound those cells seemed to recover from the initial cytotoxicity; however, after 60 h cell viability decreased again, suggesting that the extract may contain other compounds that induce additional toxicity on N 27 cells for longer exposure times.

Regarding A 549 cells, results showed that for shorter incubations only 50 µM of *S. divinorum* extract or SA was cytotoxic, while for longer periods (24, 48 and 60 h), except for 0.1 µM, all concentrations of *S. divinorum* extract or SA presented cytotoxicity. In general, our results suggest that A 549 cells were very sensitive both to *S. divinorum* extract and SA, and their toxicity is markedly dose- and time-dependent.

Data obtained for Caco 2 cells showed that 50 µM of either compound was cytotoxic in all incubation periods; however, after 60 h the cells slightly recovered from the initial

Salvia divinorum

	Cell line	Concentration (μM)			
LC_{50}	N 27	90.36 ± 0.09	22.21 ± 0.15	53.70 ± 0.21	22.97 ± 0.16
	A 549	10.63 ± 0.95	0.4155 ± 0.74	2.073 ± 0.37	20.60 ± 0.36
	Caco 2	N.D.	N.D.	N.D.	N.D.
	Hep G2	N.D.	N.D.	N.D.	N.D.
	COS 7	49.54 ± 0.53	21.68 ± 0.52	32.12 ± 0.26	21.27 ± 0.51
	Hek 293	23.99 ± 0.36	20.85 ± 0.30	17.52 ± 0.19	25.29 ± 0.25
		12h	24h	48h	60h

(N.D. – Not determined)

Salvinorin A

	Cell line	Concentration (μM)			
LC_{50}	N 27	69.43 ± 0.14	25.07 ± 0.1	67.70 ± 0.17	55.17 ± 0.07
	A 549	49.60 ± 1.47	12 ± 0.63	17.87 ± 0.29	1.27 ± 0.37
	Caco 2	N.D.	N.D.	N.D.	N.D.
	Hep G2	N.D.	N.D.	N.D.	N.D.
	COS 7	73.21 ± 0.29	31.43 ± 0.11	10.29 ± 0.12	9.17 ± 0.06
	Hek 293	22.59 ± 0.33	22.20 ± 0.33	21.85 ± 0.26	24.94 ± 0.28
		12h	24h	48h	60h

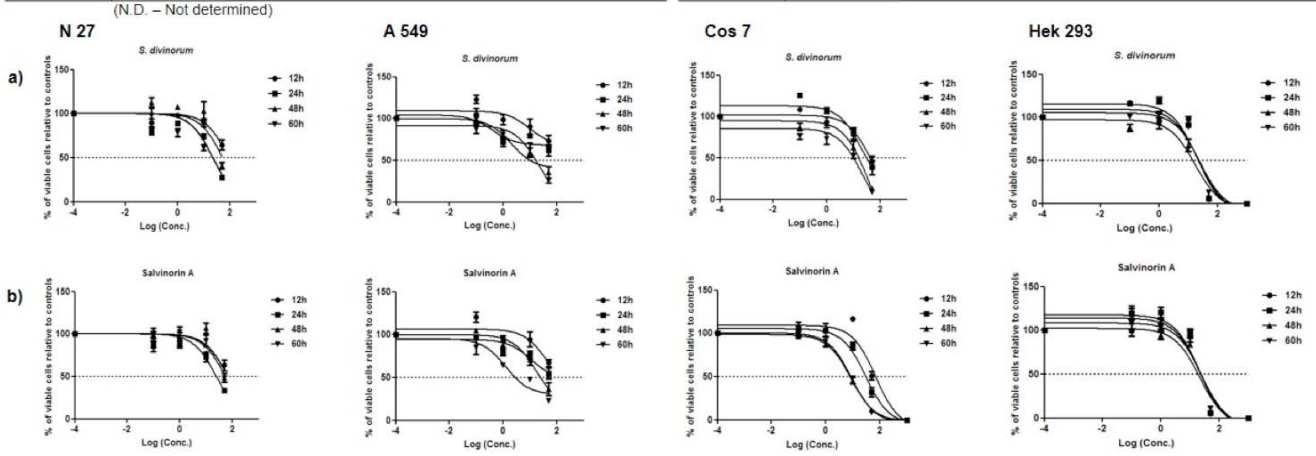

Fig. (8). Determination of LC_{50} values in cells treated with various concentrations of *S. divinorum* or salvinorin A (0.1, 1, 10 or 50 μM) for 12, 24, 48 or 60 h. MTT assays were used to determine the cell viability (%) relative to controls and, then, the IC_{50} values were determined after plotting the absorbance values *vs* drug concentrations tested using GraphPad Prism software (version 5).

cytotoxicity, suggesting the presence of other compounds in the extract that attenuate SA-promoted toxicity. Regarding the remaining concentrations, the profile was somewhat more unstable.

The results obtained for Hep G2 cells showed that 50 μM of both *S. divinorum* extract and SA promoted a decrease in cellular viability for all included periods of incubation, and in the last this effect was slightly lower for SA, when compared to the extract. Overall, and not considering the higher concentration of both *S. divinorum* extract and SA, both are only slightly cytotoxic to Hep G2 cells after 12 and 24 h of incubation, and after 48 h cells recovered, as cellular viability was not compromised; furthermore, after 60 h cellular viability has increased, when compared to controls. A possible explanation is that Hep G2 cells metabolize SA to non-cytotoxic metabolites, as toxic effects were no longer observed after 60 h of exposure.

Regarding cytotoxicity of *S. divinorum* extract or SA in COS 7 and Hek 293 cells, the results indicate that the higher concentration was cytotoxic for both cell lines in all periods of incubation, but the embryonic-derived cell line was much more sensitive. Nevertheless, the lower concentrations (0.1, 1 and 10 μM) of both compounds in Hek 293 cells indicated a lower sensitivity comparing to COS 7. Additionally, except for 50 μM, these cells showed the capability to recover from the initial cytotoxicity (induced by both *S. divinorum* extract and SA), as the percentage of cellular viability returned to values close to the controls.

From the data obtained, LC_{50} values from the cells incubated for 12, 24, 48 and 60 h with *S. divinorum* extract or SA were also determined (Fig. **8**). As expected, in Caco 2 and Hep G2 cell lines, the LC_{50} values were not determined (N.D.) because the data obtained did not fit into a statistical model. Overall, according to the LC_{50} values determined, the A 549 cell line is the most sensitive to toxicity in a time dependent manner. Notwithstanding, for shorter periods of time, both A 549 and Hek 293 cells dramatically decreased their cellular viability after exposure to *S. divinorum* extract or SA alone.

From the experiments, it was observed that depending on the cells, both *S. divinorum* extract and SA presented distinct *in vitro* toxicities, dose- and time-dependent; and, overall, with the exception for Hep G2 and Caco 2 cells, longer exposure time/ concentration have originated higher cytotoxicity.

In vivo studies with SA and/ or *S. divinorum* have revealed a variety of results, some of them inconclusive or contradictory. Also, studies on the addiction and toxicity of *S. divinorum* and SA are very scarce and limited, and, to date, there is no data regarding abuse or addiction in users of the drug. In a published survey on 500 *S. divinorum* consumers, less than 1 % had experienced feelings of addiction or dependence [50]. Also, a previous study demonstrated a weak aversive effect of SA in animals, suggesting that it is not an addictive compound [51, 52], and no cases of *S. divinorum* toxicity or deaths from overdose have been reported. Mowry *et al.* (2003) evaluated the toxicity of SA in mice by exposing them to 400-6400 μg/kg of SA daily for 2 weeks, and concluded that even at doses many times higher than those to which humans are exposed, toxicity of SA is relatively low, and histological analysis of spleen, blood, brain, liver, kidney and bone marrow did not show any histological change at any of the doses [53]. However, according to several authors, it is important to note that, in mice, a 2 week exposure is not considered chronic treatment, and thus it may

not reflect the effects of a prolonged exposure to SA. Here, it is reported, for the first time, the *in vitro* cytotoxicity of *S. divinorum* extract and SA, particularly for long-time exposures, in six widely used cell lines, suggesting that, *in vivo*, *S. divinorum*, in particular SA may also have serious implications resultant from its continuous consumption.

CONCLUSION

Considerable knowledge concerning both *S. divinorum* and SA and their effects on organisms has been achieved so far. Although SA's short duration of action has been well established, the exact mechanisms behind its metabolism, toxicity, safety, long-term effects and elimination remain, to the best of our knowledge, largely unknown. In fact, no study was carried out so far addressing these issues using *in vitro* models, or considering long periods of exposure to *S. divinorum*.

This paper clarifies, for the first time, the *in vitro* toxicity of both *S. divinorum* and SA in N 27, A 549, Caco 2, Hep G2, Cos7 and Hek 293 cells, being both dose- and time-dependent. Also, Hep G2 and, to a lesser extent, Caco 2 cells showed lower sensitivity to *S. divinorum* and SA, comparing to N 27, A 549, COS 7 and Hek 293 cell lines.

Considering the distribution and the potential therapeutic impact of prolonged SA exposure, overall, the results presented on this article update current knowledge regarding *S. divinorum*- and SA-induced toxicity and open new perspectives for research in this area.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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ABBREVIATIONS

5-HT_{2A}	=	serotonin 2A receptor
$_K$ATP channels	=	adenosine triphosphate-sensitive potassium channels
KOR	=	Kappa-opioid receptor
LC_{50}	=	concentration that kills 50 % of cells
LSD	=	lysergic acid diethylamide
MTT assay	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay
nor-BNI	=	nor-binaltorphimine
S. divinorum	=	*Salvia divinorum*
SA	=	Salvinorin A.

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