European Journal of Pharmacology 800 (2017) 96–106

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Behavioural pharmacology

22-azidosalvinorin A exhibits antidepressant-like effect in mice

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ARTICLE INFO

Keywords:
Depression
Salvinorin A
22-azidosalvinorin A
Monoamines
Adrenoceptors
κ-opioid receptor

ABSTRACT

The increasing cases of depression has made the searches for new drugs and understanding of the underligning neurobiology of this psychiatric disorder a necessity. Here, we modified the structure of salvinorin A (a known halucinogen) and investigated antidepressant-like activity of its four derivatives; 22-methylsulfanylsalvinorin A(SA1), 2-*O*-cinnamoylsalvinorin B (CSB), 22-azidosalvinorin A (SA2), and 2-*O*-(4′-azidophenylsulfonyl) salvinorin B (SA3). Prior to behavioural tests (Irwin test, open field test – OFT, forced swimming test – FST and tail suspension test – TST), SA1 was prepared by reacting salvinorin B and methylthioacetic acid with 89% yield; CSB was obtained from the reaction of salvinorin B and cinnamic acid with 92% yield; SA2 was obtained from the reaction of salvinorin B and azidoacetic acid with 81% yield; and SA3 was prepared by reacting salvinorin B with 4-azidophenylsulfonyl chloride with 80% yield. Oral treatment of mice with these derivatives (1–1000 mg/kg) did not elicit toxic sign or death. Unlike SA, SA1, CSB and SA3, treatment with SA2 (5, 10 and 20 mg/kg) decreased the immobility (TST and FST) and swimming time (FST) without altering locomotor activity in OFT. A decrease in the immobility time in TST and FST confirmed antidepressant-like property of SA2. Although p-chlorophenylalanine (serotonin depletor) or WAY100635 (selective 5-HT_{1A} receptor antagonist) did not attenuate effect of SA2, alpha-methyl-para-tyrosine (catecholamine depletor) and prazosin (selective α_1-receptor antagonist) attenuated this effect. SA2 mildly inhibited monoamine oxidase and showed affinity for α_1A, α_1B, α_1D and κ-opioid receptor subtypes. In summary, SA2 induced monoamine-mediated antidepressant-like effect.

1. Introduction

Depression is a major psychiatric disorder that affects all races, sex and better part of human lifetime around the world (Kern et al., 2012). This mood disorder casts a long and deep shadow over many facets of life. Currently, tricyclic antidepressants, selective serotonin reuptake inhibitors (SSRIs) and monoamine oxidase inhibitors (MAOIs) (Fajemiroye et al., 2016; Chaitra et al., 2011) are considered as potent antidepressants. Unfortunately, the therapeutic effect of some of these drugs are characterised by memory impairment, sedation and sexual dysfunction (Rosen et al., 1999). The searches for new agents with desirable pharmacological profiles have reinvigorated the drive for the isolation and chemical modification of natural products.

Salvinorin A (SA), a neoclerodane diterpenoid and potent selective kappa opioid agonist (Roth et al., 2002), is the main active ingredient of *Salvia divinorum*. The *S. divinorum* is an hallucinogenic plant whose use is increasing worldwide (Casselman et al., 2014). Its spiritual and recreational applications among the Mazatec Indians of northeastern Oaxaca-Mexico are associated primarily with its psychoactive effects which aid in ritual divination (Wasson, 1962, 1963). The emerging internet trading has promoted access and use of this plant species as an herbal dietary supplement or a herbal hallucinogen (Dennehy et al., 2005). SA is unique as it does not inhibit monoamine oxidase (MAO-A and B) and seems not to affect any of the receptor sites affected by other hallucinogens (Siebert, 1994; David et al., 2014). In the present study, chemical modification of SA as shown in Scheme 1 was considered.

Since SA has been reported to be inactive on MAO, adrenergic, dopaminergic and serotoninergic receptors (Siebert, 1994), battery of 50 receptors, transporters, and ion channels (Roth et al., 2002), a chemically modified SA could provide therapeutic benefit. The chemical modifications of SA seems to be a strategic approach towards the

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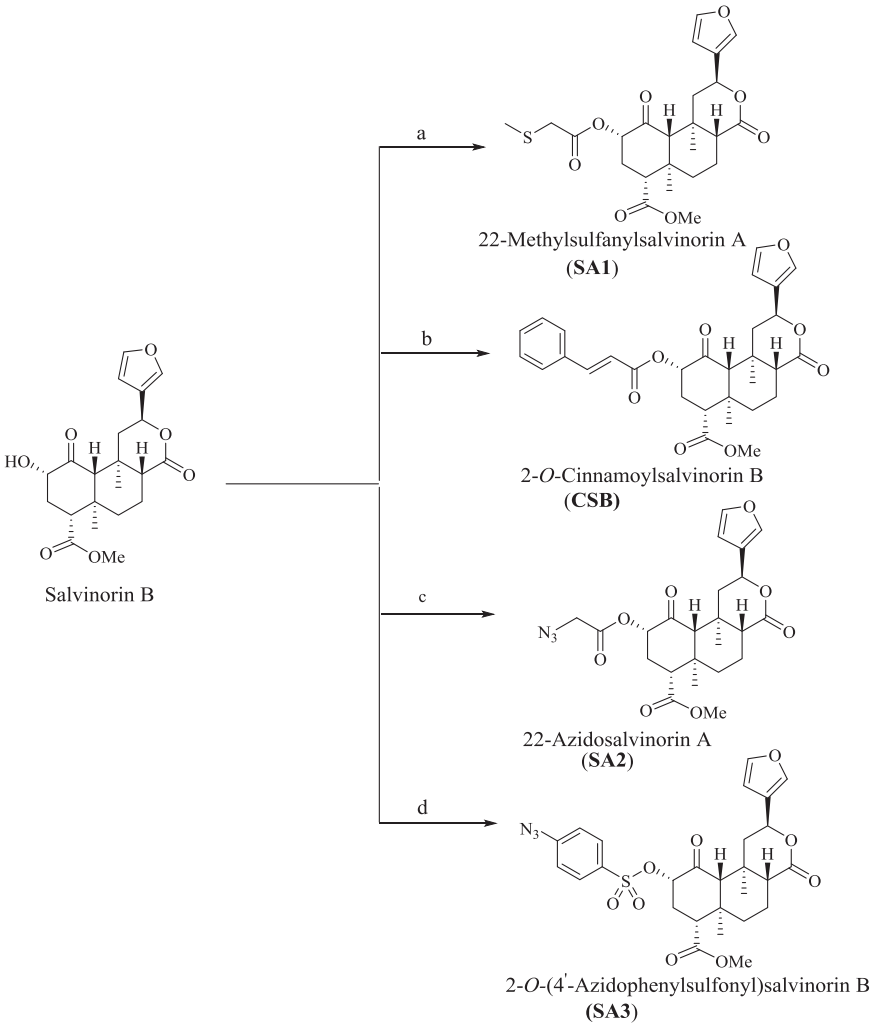
http://dx.doi.org/10.1016/j.ejphar.2017.02.031
Received 14 November 2016; Received in revised form 16 February 2017; Accepted 16 February 2017
Available online 17 February 2017



Scheme 1. Synthetic route for the designed compounds. Reagents and conditions: (a) methylthioaceticacid, DCC/DMAP, anhydrous DCM, N_2, r.t, 4 h; (b) cinnamic acid, DCC/DMAP, dry DCM, r.t, 4–5 h; (c) azidoacetic acid, DCC/DMAP, anhydrous DCM, r.t, 4 h; (d) 4-azidophenylsulfonyl chloride, Et_3N, dry DCM, N_2, r.t, 5 h. Note: Salvinorin B was formed through hydrolysis of salvinorin A.

repositioning of its kappa opioid agonist selectivity (which could be pro-depression) and halucinogenic property for therapeutic benefits. We hypothesize that the modification of SA could results in the loss of selectivity for kappa opioid receptor (a receptor that is assumed to be associated with the halucinogenic mechanism of SA) and facilitate mechanism of antidepressant action. Study of hallucinogens seems reasonable to further gain insights into the neurobiology of depression (David et al., 2014). Hence, the present study sought to synthesize, investigate antidepressant-like effect and neural mechanisms of SA derivatives.

2. Material and methods

2.1. Animals

Male Swiss mice (weighing between 25–30 g; 5–6 weeks old) housed in 15 ± 5 per cage (320×180×160 cm) were used in all the behavioural models. The animals were maintained under controlled environmental conditions (temperature 23 ± 1 °C, relative humidity 65% and 12 h light-dark cycle - lights on at 7:00 h AM) free access to water and food. Following the approval of experimental protocol (protocol number 104/08) by the Ethical and Animal Research Committee of Federal University of Goiás, experiments were conducted in accordance with the Brazilian College of Animal Experimentation guidelines for the care and use of laboratory animals as well as the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

2.2. Chemicals and drugs

Salvinorin A (starting material) was isolated from commercially available dried leaves of *Salvia divinorum*, and then converted to salvinorin B (Tidgewell et al., 2004). Other derivatives were subsequently synthesized from salvinorin B. Diazepam - DZP (Cristália, Itapira, SP, Brazil), Imipramine - IMI (Cristália, Itapira, SP, Brazil). p-chlorophenylalanine - PCPA, - N-{2-[4-(2-methoxyphenyl)–1-piperazinyl] ethyl}-N-2-pyridinylcyclohexane-carboxamide - WAY100635 or WAY (Sigma-Aldrich, St. Louis, MO, USA), Prazosin - PRAZ (Cristália, Itapira, SP, Brazil), Tween 80–2% (Polyoxyethylenesorbitan monooleate, Sigma-Aldrich, St. Louis, MO, US. For in vivo assay, drugs were dissolved in a vehicle [a mixture of 0.9% NaCl and 5% Tween-80(v/v)] and administered orally (p.o.) or intraperitoneally (i.p) in a volume of 10 ml per kg of mice body weight. All drugs were freshly prepared and protected from light. The control animals received vehicle.

2.3. Synthesis of salvinorin A derivatives

2.3.1. Procedure for the synthesis of 22-methylsulfanylsalvinorin A (SA1)

To the solution of salvinorin B (350 mg, 0.05 mmol) in DCM

(10 ml), a catalytic amount of dimethylaminopyridine (DMAP), dicyclohexylcarbodimide (DCC) (0.075 mmol) and methylthioacetic acid (0.15 mmol) were added. The reaction mixture was stirred at room temperature under N_2 for 4 h. After TLC indicated completion of the reaction, the mixture was concentrated under reduced pressure and the residue was purified by column chromatography (SiO_2; eluent: n-hexane/EtOAc) and HPLC (C18 column, MeCN–water) to yield the target product.

2.3.2. Synthesis of 2-O-cinnamoylsalvinorin B (CSB)

2-*O*-cinnamoylsalvinorin B (**CSB**) was prepared from the reaction of salvinorin B and cinnamic acid in the presence of DCC/DMAP in anhydrous dichloromethane. The detailed description has been reported in previous publication (Polepally et al., 2014).

2.3.3. Method for the synthesis of 22-azidosalvinorin A (SA2)

To the solution of salvinorin B (300 mg, 0.05 mmol) in DCM (10 ml), a catalytic amount of 4-(dimethylamino) pyridine (DMAP), 1,4-dicyclohexylcarbodimide (DCC) (0.15 mmol) and azidoacetic acid (0.15 mmol) (Srinivasan et al., 2009) were added. The reaction mixture was stirred at room temperature under N_2 for 4 h. After TLC indicated completion of the reaction, the mixture was concentrated under reduced pressure and the residue was purified by column chromatography (SiO_2; eluent: n-hexane/EtOAc) and HPLC (C18 column, MeCN–water) to yield the target product.

2.3.4. Method for the synthesis of 2-O-(4′-azidophenyl) sulfonylsalvinorin B (SA3)

To the solution of salvinorin B (300 mg, 0.05 mmol) in DCM (10 ml), a catalytic amount of triethylamine (Et_3N), and 4-azidophenylsulfonyl chloride (0.15 mmol) were added. The reaction mixture was stirred at room temperature under N_2 for 5 h. After TLC indicated completion of the reaction, the mixture was concentrated under reduced pressure and the residue was purified by column chromatography (SiO_2; eluent: n-hexane/EtOAc) and HPLC (C18 column, MeCN–water) to yield the target product.

2.3.5. Reagents and instrumental specifications

All commercially available reagents were used without further purification unless otherwise noted. All these reactions were performed under Argon atmosphere and anhydrous dichloromethane which was purchased from Sigma-Aldrich was used as solvent. The ^1H NMR spectra were recorded on a Bruker Avance–400 spectrometer using $CDCl_3$ as solvent, δ values in ppm and coupling constant *J* (Hz) assignments of ^1H resonance coupling. Thin-layer chromatography (TLC) was performed on 250 μm layer plates Whatmann PE SIL G/UV silica gel (backing polyester) plates using *n*-hexane/EtOAc, 1:1 solvent system. Spots on TLC visualized with anisaldehyde / H_2SO_4 in methanol. Column chromatography was performed with silica gel (230×400 mesh) from GA, USA. Analytical HPLC was carried out on Waters 2487 with duel λ Absorbance detector system on Phenomenex Luna-C18 column (4.6×250 mm, 5 μm) with elution at a flow rate 1.0 ml/min. All biologically evaluated compounds were found to possess more than 95% purity by HPLC.

2.4. Pharmacological approaches

2.4.1. Irwin test

Mice were subjected to Irwin test (Irwin, 1962, 1968) in the present study, to evaluate general pharmacological alterations in the course of seven days following acute administration of derivatives of salvinorin A (DSA). Changes in exploratory activity, behavioural or autonomic manifestations, signs of toxic effect or death occurrence, at different doses (DSA 1, 10, 100 or 1000 mg/kg or vehicle 10 ml/kg) and route of administrations (subcutaneous - s.c, intraperitoneal - i.p, or intragastric - p.o) were recorded.

2.4.2. Forced swimming test (FST)

Mice were exposed to a modified version of FST described by Porsolt et al. (1977) after oral administration of vehicle, DSA (5, 10 or 20 mg/kg) or IMI 15 mg/kg. Mice were later placed individually as described in the previous work (Fajemiroye et al., 2014) in a cylindrical container of 18 cm diameter filled with water to the height of 30 cm in height (total volume ($\pi r^2 h$) of 7635.06 cm^3) at 24 ± 2 °C. The initial escape-directed behaviour of mouse are often followed by a relatively passive posture. The 6-min test session was videotaped to record the swimming and immobility time prior to statistical analysis. Immobility may represent inccordination, myorelaxant, sedation or lack of interest in environmental stimulation or sign of helplessness.

2.4.3. Tail suspension test (TST)

In addition to FST, a modified version of TST validated by Steru et al. (1985) was conducted to investigate antidepressant-like effect of the derivatives of salvinorin A. Mice were randomly allocated to treatment conditions (vehicle, DSA (5, 10 or 20 mg/kg) or IMI 15 mg/kg) and suspended (using an adhesive tape placed 2 cm from the tip of the tail) at about 50 cm above the floor after 1 h of drug administartions. Mice tend to develop an immobile posture when placed in an inescapable stressful situation after initial escape-oriented movements. An increase in active escape-directed behaviours indicates antidepressant-like effect in this model. A 6-min test session was videotaped and the immobility time was later scored and analysed.

2.4.4. Open field exploratory activity

A circular open field arena with base area 62.80 cm^2 and 8 equal sectorial divisions enclosed in a 50 cm high wooden wall was used to evaluate exploratory activity of the mice as described in the previous works (Fajemiroye et al., 2014, 2015). Animals were treated with vehicle, DSA (5, 10 or 20 mg/kg, p.o), DZP (1 or 5 mg/kg, p.o) 1 h prior to 5-min test session in the open field. DZP was used as a reference drug in this model at the doses of 1 mg/kg (a dose that induces anxiolytic-like effect) and 5 mg/kg (a dose that induces sedation). Parameters like total crossing, freezing time, number of grooming and rearing activity, crossing at the centre of the open field were recorded. These parameters in the OFT which largely reflect exploratory activities of the animals could indicate stimulatory, anxiolytic, myorelaxant or sedative effects of drugs. The total crossing (measure of locomotry activities), Freezing behaviour (refers to a stress response), number of grooming and rearing activity (measure emotionality and repetitive behaviours), crossing at the centre (measure thigmotaxis, aversive behaviour and anxiolytic effect) of the open field could be altered by antidepressant treatment.

2.4.5. Mechanism of antidepressant-like effect of DSA

Mice were pre-treated (i.p) with SAL 10 ml/kg (isotonic saline solution) or AMPT 100 mg/kg (catecholamine depletor) 4 h prior to the oral administration of vehicle 10 ml/kg or DSA 5 mg/kg. In a different experiment, mice were pretreated (i.p) with SAL 10 ml/kg or PCPA 100 mg/kg (serotonin depletor) for four consecutive days prior to oral administration of vehicle or DSA 5 mg/kg. In addition, acute pretreatment of mice with PRAZ 1 mg/kg (α_1- adrenoceptor antagonist), WAY 0.3 mg/kg (a selective antagonist of 5-HT_{1A} receptor) or SAL 10 ml/kg prior to the oral administration of vehicle 10 ml/kg or DSA 10 mg/kg (30 mins interval between i.p pretreatment and p.o treatment) was followed by behavioural testing - FST (1 h interval between p.o treatment and FST). The regimen of PCPA depleted about 58.8% of endogenous storage of serotonin content while AMPT reduced 55.2% of dopamine and 51.7% of noradrenaline levels.

2.4.6. Effect of DSA on recombinant human MAO-A and -B

Kynuramine (KYN) deamination assay was performed in 96-well plates as described earlier to investigate the effect of DSA on the activities of recombinant human MAO-A and –B (Blier and de

Montigny, 1994). Varying concentrations of DSA, clorgyline or deprenyl (to determine the IC_{50} values) were tested on KYN (substrate) incubated with recombinant human MAO-A and -B. the apparent K_m values (the substrate concentration at half V_{max}) for substrate binding that was reported previously was used to determine the KYN concentration of 80 and 50 μM for MAO-A and -B, respectively (Wouters, 1998). Inhibition of enzymatic activity was calculated as percent of product formation compared to the corresponding enzyme-substrate reaction without inhibitors. The reactions were carried out in. Incubation mixtures contained 5 μg/ml of MAO-A or 12.5 μg/ml of MAO-B in 50 μL of 0.1 M potassium phosphate buffer at pH 7.4. The compounds were dissolved in DMSO to yield total reaction volume of 200 μL at final DMSO concentration of 1.0% in the reaction mixture. The KYN and DSA, clorgyline or deprenyl were pre-incubated for 10 min at 37 °C prior to addition of MAO-A or MAO-B. The assay plates were incubated at 37 °C to observe enzymatic reactions for 20 min and stopped by the addition of 75 μL of 2N NaOH. The product formed (4-hydroxyquinoline) was detected fluorometrically by SpectraMax M5 fluorescence plate reader (Molecular Devices, Sunnyvale, CA) using the Soft Max Pro program (with an excitation and emission wavelength of 320 nm and 380 nm, respectively).

2.4.7. α_1 adrenoceptors binding assay

Cell culture, transient transfection and radioligand binding assay were carried out as described by Chen et al. [26]. The membranes with α_1A, α_1B and α_{1D}-AR subtype were incubated with [3H] prazosin (0.1–10 nM) in a total volume of 100 μL. In order to carry out competition binding experiments, 1–5 μg of α_1A and α_1B -ARs and 5–20 μg of α_1D -AR membranes were incubated with 200 pM of [3H] prazosin prior to addition of increasing concentrations of SA2 in a total volume of 100 μL. Experiments were carried out in triplicate. Ki values were calculated as described previously (Chen et al., 2013) according to the equation of Cheng and Prusoff. Ki=IC50/1+([L]/KD) when [L] and KD are the radioligand concentration and dissociation constant, respectively.

2.4.8. Opioid receptors binding assays

The binding assays of kappa-opioid (KOR), mu-opioid (MOR) and delta opioid (DOR) receptor were performed as previously detailed (Eyal et al., 2013; Butelman and Kreek, 2015).

2.5. Statistical analysis

The data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test or two-way ANOVA followed by Bonferroni's post hoc test. Data are expressed as means ± S.E.M. and differences are considered statistically significant when P-values are less than 0.05. All analysis were carried out using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. ^1H, ^{13}C NMR and MS data for compounds

NMR and MS data were obtained for all products purified by column chromatography and preparative HPLC.

3.1.1. 22-methylsulfanylsalvinorin A(SA1)

White amorphous powder, ^1H NMR (400 MHz, $CDCl_3$): δ 7.42 (s, 1H), 7.40 (s, 1H), 6.39 (s, 1H), 5.54 (dd, *J* =5.2, 11.4 Hz, 1H), 5.23 (dd, *J* =7.6, 12.6 Hz, 1H), 3.74 (s, 3H), 2.76 (dd, *J* =8.6, 8.6 Hz, 1H), 2.54 (dd, *J* =5.2, 13.4 Hz, 1H), 2.38–2.33 (m, 2H), 2.20–2.16 (m, 2H), 2.10–2.06 (m, 1H), 1.81 (ddd, *J* =3.0, 3.0, 10.4 Hz, 1H), 1.71–1.55 (m, 3H), 1.47 (s, 3H), 1.13 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 201.82, 171.89, 170.47, 167.12, 142.18, 139.57, 124.21, 109.32, 74.65, 71.80, 63.58, 53.22, 52.58, 51.92, 51.07, 42.74, 41.88, 40.81, 37.98, 35.28, 30.57, 18.09, 16.50, 15.10, 14.19. HRESIMS (*m/z*): $[M+H]^+$ calculated for $C_{24}H_{31}O_8S$, 479.1661; found, 479.1658 HPLC t_R =12.854 min; purity =95.6%; yield =89%.

3.1.2. 2-O-cinnamoylsalvinorin B (CSB)

White amorphous powder with 92% yield. The ^1H NMR and ^{13}C NMR data of **CSB** have been reported in the previous article (Polepally et al., 2014).

3.1.3. 22-azidosalvinorin A (SA2)

White amorphous powder, ^1H NMR (400 MHz, $CDCl_3$): δ 7.42 (s, 1H), 7.41 (s, 1H), 6.39 (s, 1H), 5.54 (dd, *J* =5.2, 11.4 Hz, 1H), 5.20 (dd, *J* =7.6, 12.6 Hz, 1H), 3.97 (s, 2H), 3.74 (s, 3H), 2.78 (dd, *J* =8.6, 8.6 Hz, 1H), 2.52 (dd, *J* =5.2, 13.4 Hz, 1H), 2.38–2.34 (m, 2H), 2.21–2.16 (m, 2H), 2.10–2.07 (m, 1H), 1.82 (ddd, *J* =3.0, 3.0, 10.4 Hz, 1H), 1.74–1.68 (m, 3H), 1.46 (s, 3H), 1.14 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 201.79, 172.01, 171.86, 167.13, 142.15, 140.32, 125.03, 109.82, 74.65, 71.80, 63.58, 54.89, 53.22, 52.58, 51.92, 51.07, 42.74, 41.88, 40.81, 37.98, 35.28, 30.57, 18.09, 16.50, 15.10. HRESIMS (*m/z*): $[M+H]^+$ calculated for $C_{23}H_{28}N_3O_8$, 474.1798; found, 479.1792 HPLC t_R =24.312 min; purity =96.4%; yield =81%.

3.1.4. 2-O-(4´-azidophenylsulfonyl)salvinorin B (SA3)

White amorphous powder, ^1H NMR (400 MHz, $CDCl_3$): δ 7.92 (d, *J* =7.8 Hz, 1H), 7.54 (d, *J* =7.8 Hz, 1H), 7.44 (s, 1H), 7.43 (s, 1H), 6.40 (s, 1H), 5.54 (dd, *J* =5.2, 11.4 Hz, 1H), 5.03 (dd, *J* =7.6, 12.6 Hz, 1H), 3.73 (s, 3H), 3.50 (s, 2H), 2.74 (dd, *J* =8.6, 8.6 Hz, 1H), 2.44–2.35 (m, 2H), 2.19–2.15 (m, 1H), 2.10 (s, 1H), 2.09–2.05 (m, 2H), 1.81 (ddd, *J* =3.0, 3.0, 10.4 Hz, 1H), 1.73–1.62 (m, 3H), 1.44 (s, 3H), 1.11 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 201.24, 143.80, 139.63, 135.45, 132.18, 129.13, 126.13, 125.12, 108.49, 81.09, 71.82, 63.98, 57.30, 53.20, 52.08, 51.19, 43.16, 41.92, 37.94, 35.52, 31.99, 18.06, 16.48, 15.20. HRESIMS (*m/z*): $[M+H]^+$ calculated for $C_{27}H_{30}N_3O_9S$, 572.1625; found, 572.1619 HPLC t_R =12.184 min; purity =98.4% yield =80%.

3.2. General behavioural alterations induced by DSA

In Irwin test (Table 1), SA1, CSB or SA3 elicited sedation at the dose of 100 and 1000 mg/kg while SA2 reduced locomotion after 15 mins of i.p or s.c administration. An increase in exploratory activities was observed after 30 mins of SA1 or SA2 i.p and s.c administration at doses 1 and 100 mg/kg. Oral administration of SA2 increased exploratory activity after 30 mins at the doses of 100 and 1000 mg/kg while the same doses of SA2 administered through s.c or i.p route reduced exploratory activity. Subcutaneous or intraperitoneal administration of SA1, CSB, SA2, SA3 induced sedation, loss of grip strength and ataxia. SA2 100 and 1000 mg/kg induced sedation while SA1, CSB, SA3 induced sedation, loss of grip strength, ataxia in the same doses. After seven days of observation, all mice recovered from DSA-induced behavioural alterations without weight gain, sign of toxicity or death. No significant alteration was recorded with SA (data not shown).

3.3. Effects of DSA treatment on mice subjected to the TST

Oral administration of SA [F (4, 30) =8.14, p > 0.05, Fig. 1A], SA1 [F (4, 30) =4.26, P > 0.05, Fig. 1B], CSB [F (4, 30) =8.02, P > 0.05, Fig. 1C], and SA3 [F (4, 30) =4.53, P > 0.05, Fig. 1E] did not alter immobility time (at all doses tested) in the TST. In contrary, SA2 10 mg/kg [F (4, 30) =7.95, P < 0.05, Fig. 1D] and SA2 20 mg/kg [F (4, 30) =7.95, P < 0.001, Fig. 1D] reduced the immobility time.

Table 1
Changes elicited by subcutaneous (s.c), intraperitoneal (i.p) or oral (p.o) administration of salvinorin A derivatives (SA1, CSB, SA2, and SA3).

Observation time after administration	Dose (mg/kg)	Administration routes and observations	
		s.c or i.p	p.o
15 min	1, 10	SA1, CSB, SA2, SA3: N	SA1, CSB, SA2, SA3: N
	100, 1000	SA1, CSB, SA3: Sedation SA2: Reduced locomotion	
30 min	1, 10	SA1, SA2: Increase in exploratory activities CSB, SA3: N	
	100, 1000	SA1, CSB, SA3: Sedation SA2: Reduced exploration	SA2: Increase in exploratory
1 h	1, 10	SA1, CSB, SA2, SA3: N	SA2: Sedation SA1, CSB, SA3: ataxia
	100, 1000	SA1, CSB, SA2, SA3: Sedation, loss of grip strength, Ataxia	SA1, SA2: sedation, loss of grip strength CSB, SA3: Ataxia
Day-7	Mice recovered from the effects of SA1, CSB, SA2, and SA3 without weight gain, sign of toxicity or death.		

N - Non occurrence or absence of significant behavioural alteration as compared to vehicle treated group.

3.4. Effects of SA and SA1 treatment on mice subjected to the FST and OFT

Unlike SA (Fig. 2), oral administration of SA1 10 mg/kg increased swimming time [F (4, 30) =6.91, P < 0.001, Fig. 3A] without altering immobility time [F (4, 30) =3.85, P > 0.05, Fig. 3B] in the FST. In the OFT, unlike DZP, SA1 increased total crossing [at the dose 10 mg/kg; F (5, 36) =12.24, P < 0.001, Fig. 3C] and crossing at the centre [at doses 5, 10 or 20 mg/kg; F (5, 36) =7.01, P < 0.05; F (5, 36) =7.01, P < 0.001 and F (5, 36) =7.01, P < 0.001, respectively; Fig. 3F]. The freezing time [F (5, 36) =6.35, P > 0.05, Fig. 3D] and number of rearing [F (5, 36) =5.57, P > 0.05, Fig. 3E] remained statistically unaltered.

3.5. Effects of CSB treatment on mice subjected to the FST and OFT

Except for the oral administration of CSB 10 mg/kg that increased

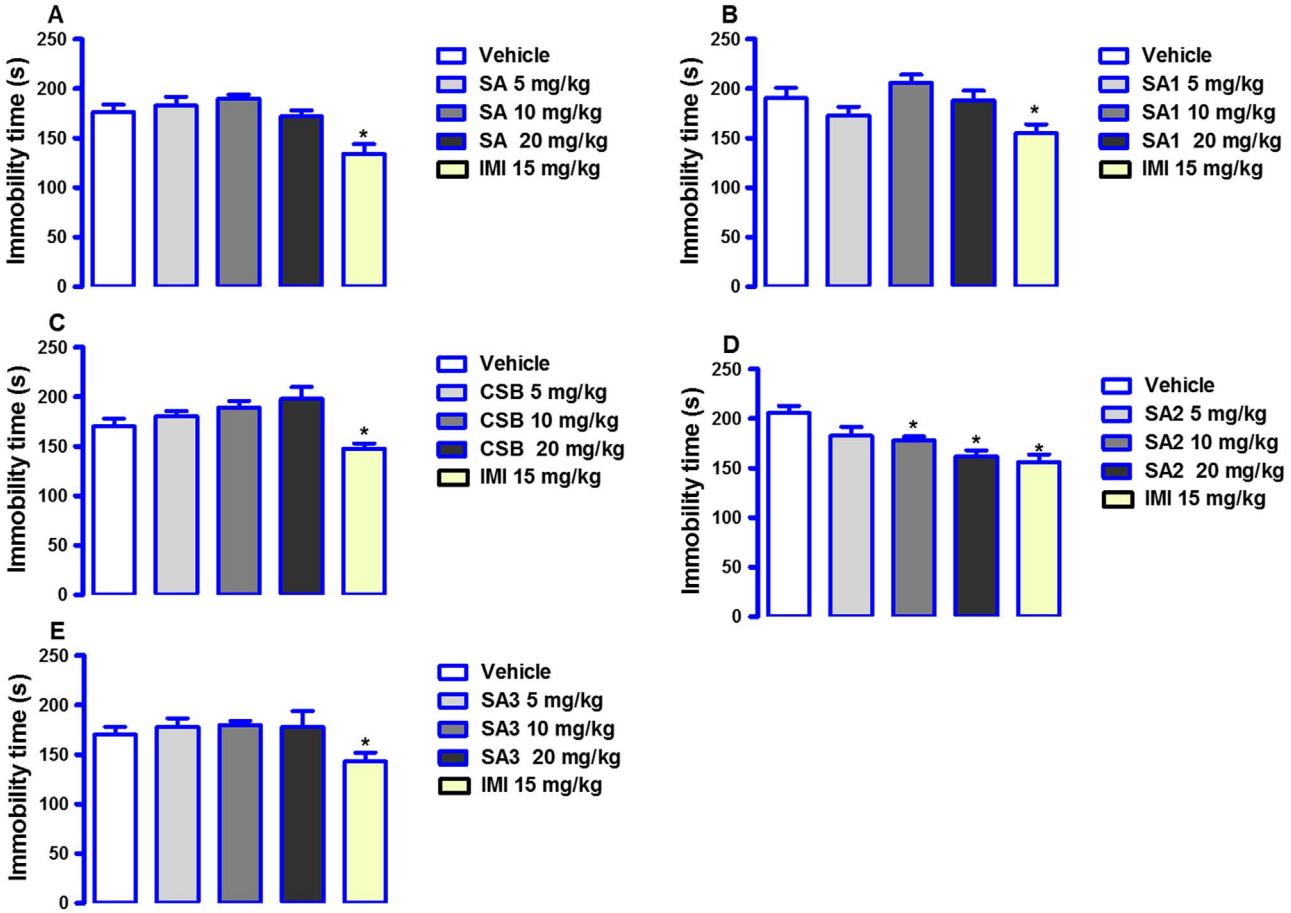


Fig. 1. The effect of oral administration of Salvinorin A - SA (A), 22-methylsulfanylsalvinorin A - SA1 (B), 2-*O*-cinnamoylsalvinorin B - CSB (C), 22-azidosalvinorin A - SA2 (D), 2-*O*-(4′-azidophenylsulfonyl) salvinorin B - SA3 (E) at the doses of 5, 10 or 20 mg/kg and imipramine (IMI) 15 mg/kg or vehicle 10 ml/kg on the immobility time in the tail suspension test (TST) (one-way ANOVA followed by Dunnett's test).

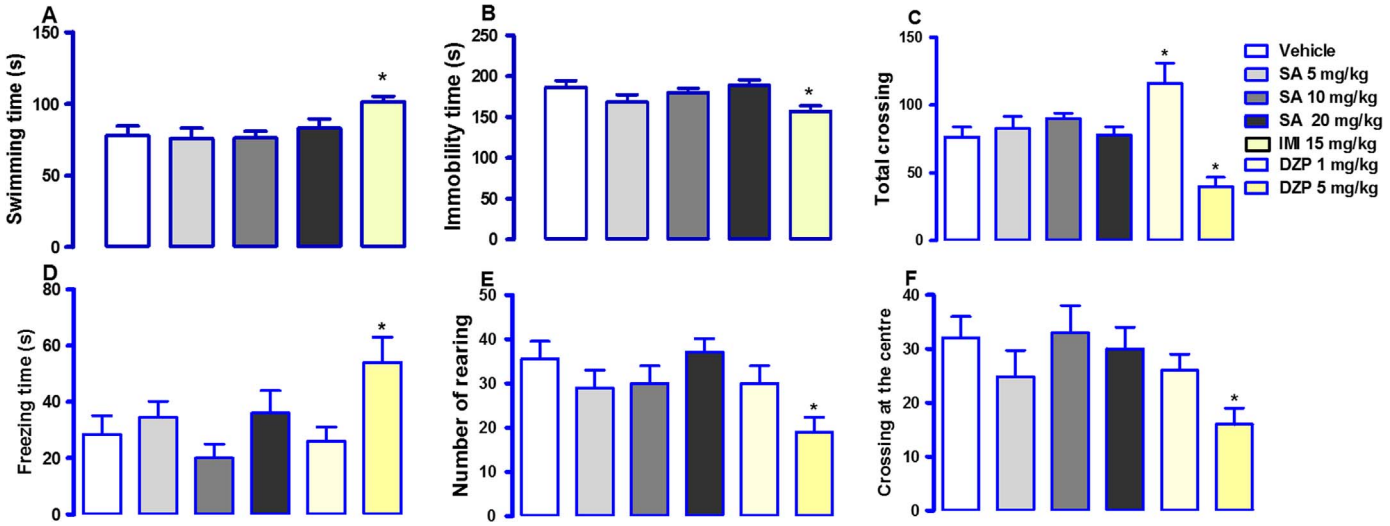


Fig. 2. The effect of oral administration of vehicle, Salvinorin A (SA –5, 10 and 20 mg/kg) or imipramine (IMI) 15 mg/kg on the swimming time (A) and immobility time in the FST (B). Fig. 2C, D, E and F show the effect of oral administrations of vehicle 10 ml/kg, SA 5, 10, 20 mg/kg, diazepam (DZP) 1 or 5 mg/kg on the total crossing, freezing time, number of rearing and crossing at the centre of the open-field, respectively. Each column represents the mean ± S.E.M. of 10 animals. *P < 0.05 vs vehicle treated group (one way ANOVA followed by Dunnett´s test).

total crossing [F (5, 36) =15.61, P < 0.05, Fig. 4C] and number of rearing [F (5, 36) =9.62, P > 0.05, Fig. 4E] in OFT, the swimming time [F (4, 30) =2.92, P > 0.05, Fig. 4A], immobility time [F (4, 30) =4.84, P > 0.05, Fig. 4B] in the FST, freezing time in OFT [F (5, 36) =10.15, P > 0.05, Fig. 4D] and crossing at the centre [F (5, 36) =15.61, P > 0.05, Fig. 4F] remained unaltered significantly.

3.6. Effects of SA2 treatment on mice subjected to the FST and OFT

Oral administration of SA2 5, 10 or 20 mg/kg [F (4, 30) =4.50] increased swimming time in the FST (P < 0.05, Fig. 5A). The immobility time was decreased in the FST at doses 10 and 20 mg/kg [F (4, 30) =3.23, P < 0.05, Fig. 5B]. SA2 did not alter total crossing [F (5, 36) =10.64, P > 0.05, Fig. 5C] and freezing time [F (5, 36) =11.14, P > 0.05, Fig. 5D]. The number of rearing was reduced at dose 20 mg/kg [F (5, 36) =9.25, P < 0.05, Fig. 5E] while crossing at the centre was increased at doses 5, 10 and 20 mg/kg [F (5, 36) =5.72, P < 0.001, Fig. 5F].

3.7. Effects of SA3 treatment on mice subjected to the FST and OFT

Oral administration of SA3 increased swimming time at dose 20 mg/kg [F (4, 30) =5.18, P < 0.05, Fig. 6A] in the FST and reduced number of rearing at doses 10 and 20 mg/kg [F (5, 36) =9.08, P < 0.05, Fig. 6D] in the OFT. There was no significant alteration in immobility time [F (4, 30) =4.52, P > 0.05, Fig. 6B] in the FST, total crossing [F (5, 36) =9.07, P > 0.05, Fig. 6C], freezing time [F (5, 36) =13.12, P > 0.05, Fig. 6D] and crossing at the centre [F (5, 36) =4.21, P > 0.05, Fig. 6E] in the OFT.

3.8. Effects of AMPT, PCPA PRAZ or WAY pretreatments and SA2 treatment

Fig. 7 showed the effect of AMPT, PCPA, PRAZ or WAY pretreatment (independent variable) prior to vehicle or SA2 treatment (independent variable) on the (A) swimming time (dependent variable) and (B) immobility time (dependent variable) in the FST. Pairwise

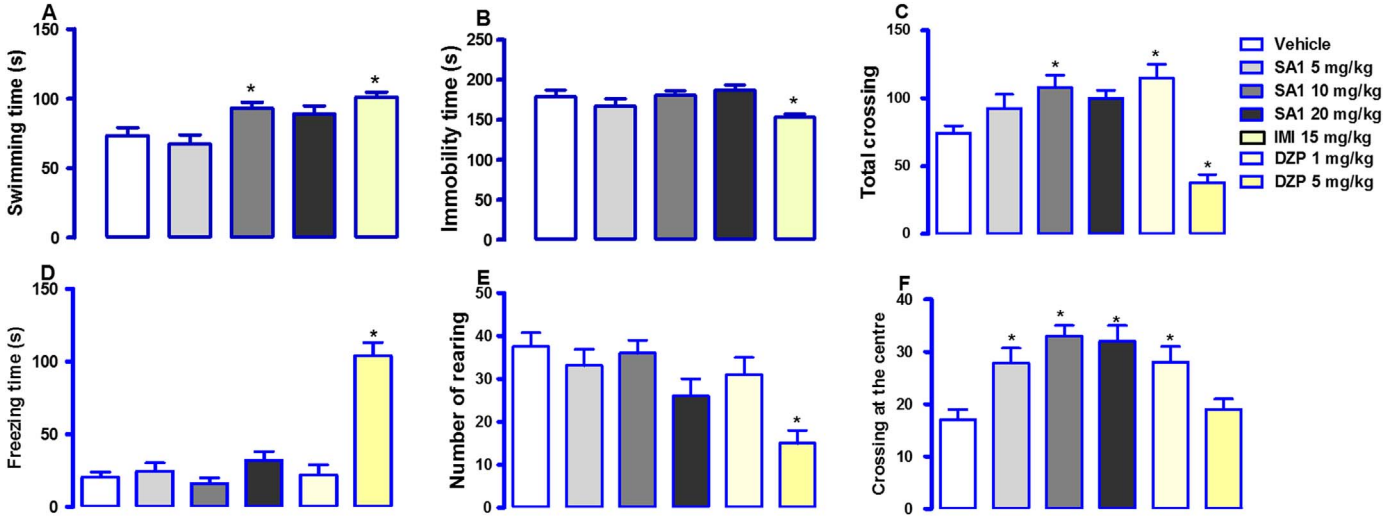


Fig. 3. The effect of acute oral administration of vehicle, 22-methylsulfanylsalvinorin A (SA1–5, 10 and 20 mg/kg) or imipramine (IMI) 15 mg/kg on the swimming time (A) and immobility time in the FST (B). Fig. 2C, D, E and F show the effect of oral administrations of vehicle 10 ml/kg, SA1 (5, 10, 20 mg/kg), diazepam (DZP) 1 or 5 mg/kg on the total crossing, freezing time, number of rearing and crossing at the centre of the open-field, respectively. Each column represents the mean ± S.E.M. of 10 animals. *P < 0.05 vs vehicle treated group (one way ANOVA followed by Dunnett´s test).

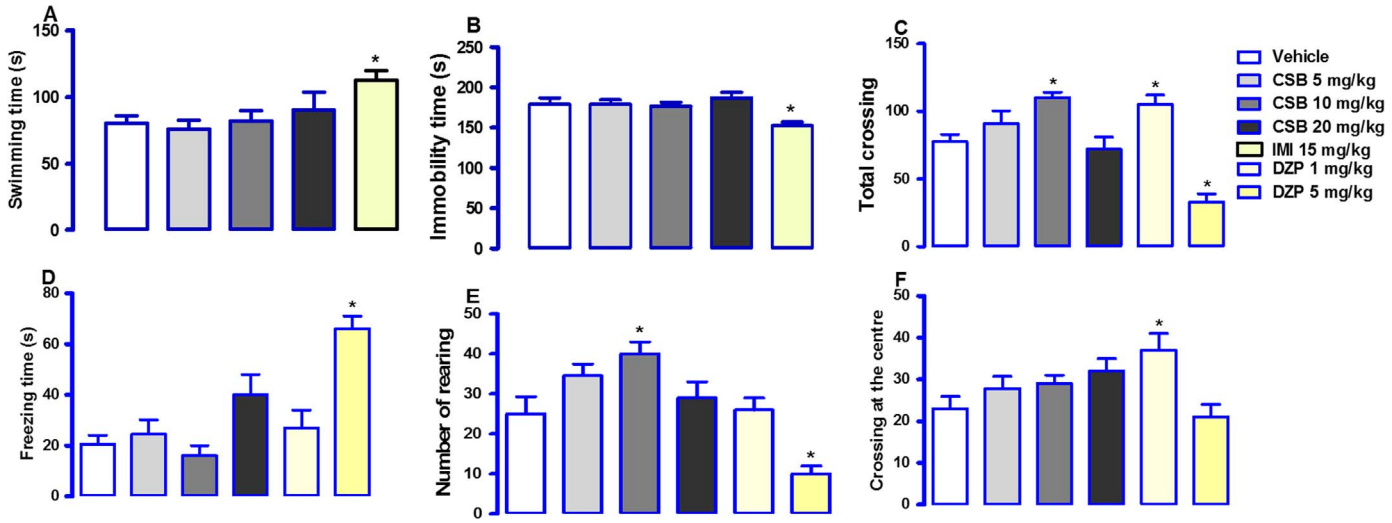


Fig. 4. The effect of acute oral administration of vehicle, 2-*O*-cinnamoylsalvinorin B (CSB −5, 10 and 20 mg/kg) or imipramine (IMI) 15 mg/kg on the swimming time (A) and immobility time in the FST (B). Fig. 2C, D, E and F show the effect of oral administrations of vehicle 10 ml/kg, CSB 5, 10, 20 mg/kg, diazepam (DZP) 1 or 5 mg/kg on the total crossing, freezing time, number of rearing and crossing at the centre of the open-field, respectively. Each column represents the mean ± S.E.M. of 10 animals. *P < 0.05 vs vehicle treated group (one way ANOVA followed by Dunnett´s test).

comparisons with Bonferroni post hoc test showed antidepressant-like effect of SA2 through a significant increase in swimming time and reduction in immobility time (i.e SAL+vehicle vs SAL+SA2, respectively, P < 0.05). Pretreatment with AMPT or PRAZ attenuated antidepressant-like effect of SA2 (i.e SAL+SA2 vs AMPT+SA2, P < 0.05). In contrast, pretreatment with PCPA or WAY did not attenuate antidepressant-like effect of SA2 (i.e SAL+vehicle versus PCPA+SA2 or WAY +SA2, respectively, P > 0.05). Pretreatments with AMPT, PCPA, PRAZ or WAY, prior to vehicle administration did not alter animal behaviour (i.e AMPT+vehicle, PCPA+vehicle, PRAZ+vehicle, WAY+vehicle versus SAL + vehicle; P > 0.05).

3.9. Effects of SA2 on the activities of monoamine oxidase (MAO) A and B

SA did not inhibit MAO A and B (8B and E, respectively). SA2 showed mild inhibition of both MAO A and B in vitro (Fig. 8C and F, respectively). Both clorgyline and deprenyl showed potent inhibition of MAO A and B, respectively (Fig. 8A and D).

3.10. Binding affinity of 22-azidosalvinorin at α_1A, α_1B and α_1D adrenergic receptors-AR

The SA2 affinity for α_1A, α_1B and α_{1D}-AR subtypes did not shows significantly difference (P > 0.05, Table 2).

3.11. KOR, MOR and DOR binding assay

The affinity of SA2 at KOR in nM =8.1 ± 1.2, MOR in nM = > 10000 and DOR in nM =4230 ± 62. SA2 seems to have lower affinity for KOR as compared to Salvinorin A (affinity at KOR in nM =1.2 ± 0.4).

4. Discussion

Salvinorin A has been reported as the main active ingredient of the hallucinogenic plant *Salvia divinorum* (Siebert, 1994; Valdés et al., 1983, 2001). Contemporary pharmacological studies are attempting to discern the therapeutic potential of SA and its mechanism of actions. Salvinorin A has several effects that complicate its clinical application,

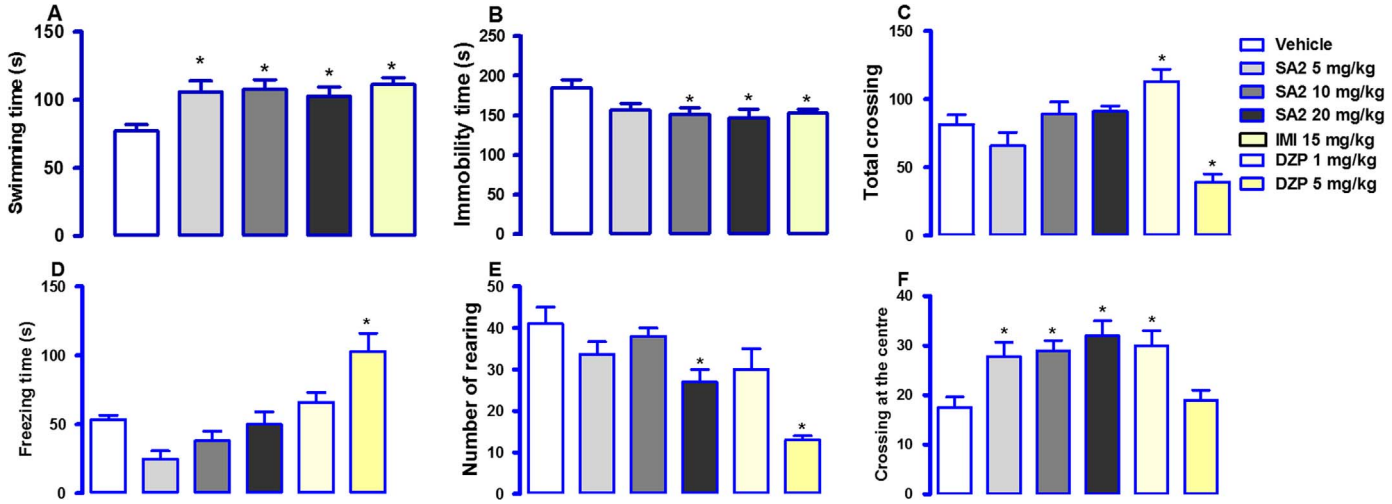


Fig. 5. The effect of acute oral administration of vehicle, 22-azidosalvinorin A (SA2−5, 10 and 20 mg/kg) or imipramine (IMI) 15 mg/kg on the swimming time (A) and immobility time in the FST (B). Fig. 2C, D, E and F show the effect of oral administrations of vehicle 10 ml/kg, SA2 (5, 10, 20 mg/kg), diazepam (DZP) 1 or 5 mg/kg on the total crossing, freezing time, number of rearing and crossing at the centre of the open-field, respectively. Each column represents the mean ± S.E.M. of 10 animals. *P < 0.05 vs vehicle treated group (one way ANOVA followed by Dunnett´s test).

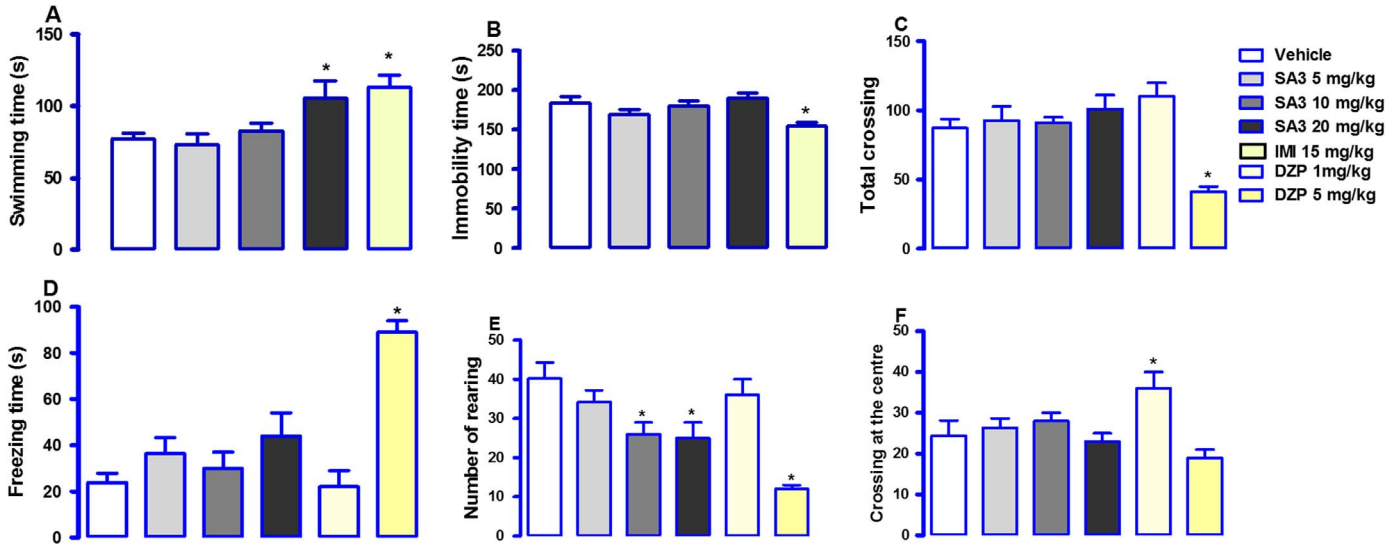


Fig. 6. The effect of acute oral administration of vehicle, 2-*O*-(4′-azidophenylsulfonyl) salvinorin B (SA3–5, 10 and 20 mg/kg) or imipramine (IMI) 15 mg/kg on the swimming time (A) and immobility time in the FST (B). Fig. 2C, D, E and F show the effect of oral administrations of vehicle 10 ml/kg, SA3 (5, 10, 20 mg/kg), diazepam (DZP) 1 or 5 mg/kg on the total crossing, freezing time, number of rearing and crossing at the centre of the open-field, respectively. Each column represents the mean ± S.E.M. of 10 animals. *P < 0.05 vs vehicle treated group (one way ANOVA followed by Dunnett´s test).

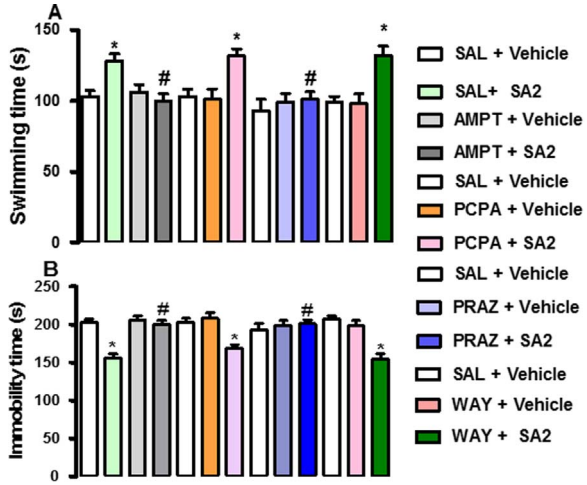


Fig. 7. Alteration in swimming (A) and immobility (B) time following pretreatment with SAL (saline solution) 10 ml/kg, AMPT (*α*-methyl-p-tyrosine), PCPA (*p*-chlorophenylalanine methyl ester) 100 mg/kg, PRAZ (prazosin) 1 mg/kg or WAY (WAY100635) 0.3 mg/kg prior to oral treatment with vehicle 10 ml/kg or SA2 10 mg/kg. Data are expressed as mean ± S.E.M., n=5 (two way ANOVA followed by Bonferroni test). *P < 0.05 vehicle treated group versus other experimental groups; #P < 0.05 SAL+SA2 versus AMPT+SA2 (two way ANOVA followed by Bonferroni test).

including dissociative and hallucinogenic effects, thought to be due to high efficacy KOR agonism (Johnson et al., 2011; Ranganathan et al., 2012; MacLean et al., 2013). In addition, salvinorin A has short duration of action in humans and non-human primates, possibly as a result of p-glycoprotein blood-brain barrier efflux transporter (Hooker et al., 2008; Butelman et al., 2012a; Johnson et al., 2011). Chemical modification was considered in the present study towards changing pharmacological profile of salvinorin A.

The chemical modification approach is supported by the previous structure-activity-studies of SA (Polepally et al., 2014). Chavkin et al. (2004) revealed that the 2-position of SA is crucial for activity. This explains the inactivity of salvinorin-B, which differs from salvinorin-A by loss of the 2-acetoxy group (Chavkin et al., 2004). The rapid hydrolysis of SA by esterase to form salvinorin-B has been suggested to be responsible for short duration of SA's action (Roth et al., 2004). The short acting effect of SA (even in encapsulated form) and its adverse effects makes its chemical modification pertinent in the current study.

Behavioural profiling of drug action represents integrated sum of activities being mediated by the nervous system (Moser, 2011). A general pharmacological screening such as Irwin test could tap different behavioural repertoires. The dose- and time-related effects of four derivatives (following acute administrations through s.c, i.p, or p.o routes) on mice physical appearance, signals and reactions to various stimuli were evaluated in a soundproof room. As a first tier of preclinical testing, this test is considered core battery studies that includes specifically motor activity, grip strength (Meyer et al., 1979), behavioural changes, body temperature, sensory and motor reflex responses. Some autonomic endpoints like salivation, lacrimation and pupil size could also be assessed (Moser, 2011).

Irwin screening has been considered as the systematic evaluation of mice to determine CNS side effects of drugs (Irwin, 1962, 1968). This test permits the observation of drug induced-behavioural change, report early signs of toxic effect or death occurrence in addition to variations in pharmacological responses to different route of drug administrations. Sensory testing that involves simple reflexes through tail grasping, pinna reflex or evaluation of the motor response to auditory and nociceptive stimuli did not show any significant alterations. There was no record of tremor, convulsions, ptose, straub or other signs of abnormalities. Considering the focus of this study on the antidepressant-like property of DSA, close attention was paid to the neuromuscular coordination through muscle tone, extensor strength, hindlimb and forelimb grip strength, righting or proprioceptive responses. Oral administration of these derivatives seems to be a secure route of administration as it only elicited a mild motor incoordination in mice at a very high dose as compared to subcutaneous and intraperitoneal routes. Hence, oral administration was adopted in subsequent studies (TST, FST and OFT). The TST and FST are well-validated models of depression (Petit-Demouliere et al., 2005; Can et al., 2012) to induce despair-like behaviour. There is a significant correlation between clinical potency and effectiveness of antidepressants in these models (Xiaosu et al., 2014).

Like SA, none of the SA1, CSB and SA3 showed promising antidepressant-like effect in both TST and FST. Although SA1 5 mg/kg increased swimming time (without altering immobility time), total crossing and crossing at the centre of open field, it is uncertain if an increase in swimming time is associated with antidepressant or

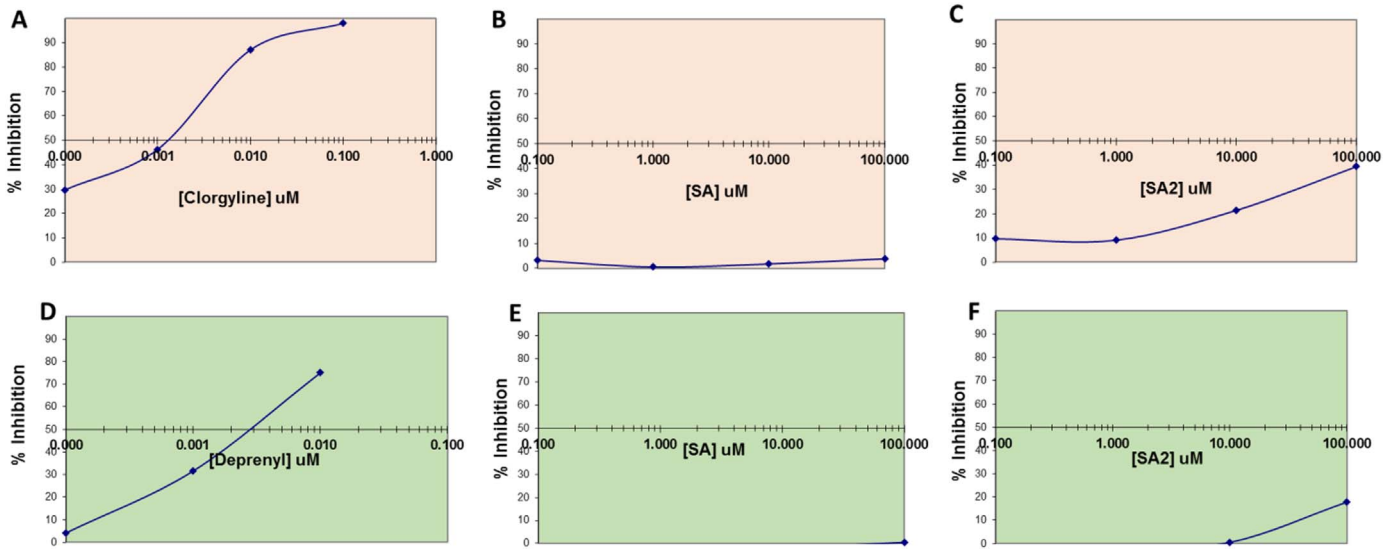


Fig. 8. In vitro measurement of the inhibitory effect of clorgyline, SA or SA2 on monoamine oxidase (MAO) A (Fig. A, B and C, respectively) and MAO B (Fig. D, E and F, respectively) catabolic activity. Data are expressed as percentage of mean enzymatic activity.

Table 2
The α_1A, α_1B and α_1D adrenergic receptors binding affinities of 22-azidosalvinorin (SA2).

	α_1A		**α_1B**			**α_1D**
	Parameters					
Test compound	p*Ki*[a]	*Ki*[b] (nM)	p*Ki*[a]	*Ki*[b] (nM)	p*Ki*[a]	*Ki*[b](nM)
SA2	6.61 ± 0.04	27,001	6.59 ± 0.11	31,110	6.26 ± 0.09	29,577

The data are expressed as the mean ± standard error of triplicate assays.
[a] Equates to the negative log of the *Ki* value.
[b] Concentration of ligand required to occupy 50% of all receptors if no radioligand were present.

stimulating property of this derivative. SA3 seems to induce similar behavioural changes as SA1 in the FST at 20 mg/kg. In the OFT, SA3 only reduced number of rearing activities. Although swimming and immobility time seems to be an opposing behavioural activities, an increase in swimming is not often directly proportional to a reduction in immobility time as mice could be involved in other activities like jumping, climbing among other escape directed behaviour. In contrary, SA2 elicited a significant increase in swimming time (FST) and reduction in the immobility time (in both FST and TST). An increase in swimming time and reduction in immobility time a5re considered as indicator of antidepressant-like property in the previous study (Fajemiroye et al., 2014). Further pharmacological studies and possible clinical trial of new DSA are expected in the future.

The specificity of the observed antidepressant-like effect of SA2 was examined through the locomotor activity of mice. In the open field, there were no changes in total crossing, freezing time and rearing activities. Hence, no evidence indicating stimulatory effect or motor incoordination in mice at the doses that significantly induced antidepressant-like effect. As FST or TST models are associated with some level of aversion that could influence performance of animals, an increase in the crossing at the centre of open field indicate anti-aversion property of SA2.

In order to evaluate possible involvement of monoaminergic mechanisms in the antidepressant-like effect of SA2, animals were pretreated with AMPT (catecholamine depletor), PCPA (serotonin depletor), PRAZ (α_1-adrenoceptor antagonist) and WAY100635 (a selective 5-HT_{1A} receptor antagonist). PCPA treatment regimen reduced 58.8% of serotonin content while AMPT reduced 55.2% of dopamine and 51.7% of noradrenaline levels. The experimental protocols for the catecholamine depletion with AMPT in this study were similar to those used in previous studies that reduces 57% of dopamine and 53% of noradrenaline levels (Mayorga et al., 2001). The regimen of PCPA in this study has been reported to deplete about 60% of serotonin content without altering catecholamine levels (Kwon et al., 2010; Redrobe et al., 1998a, 1998b). In the present study, AMPT and PRAZ attenuated the effect of SA2, thereby suggesting involvement of catecholamine.

As antagonism of the α_1-AR blocked the antidepressant-like effect of SA2, binding assay was carried out to further elucidate the involvement of this receptor and its subtypes - α_1A, α_1B and α_1D (Sarma et al., 2005). As we presumed hypoactivity of α_1-AR to be associated with depressive like behaviour, we hypothesized that direct activation of α_1-AR by SA2 could facilitates adrenergic stimulation, signaling (including phosphorylation, desensitization, trafficking, and transcription) and antidepressant-like phenotype in mice. The noradrenergic system is involved in the regulation of mood (Cunha et al., 2013). The roles of norepinephrine transporters, expression and activation of adrenergic receptors are critical to antidepressant mechanisms. The α-AR modulate norepinephrine release (Rump and Majewski, 1987). Hence, they are potential targets for antidepressant drug development (Cunha et al., 2013). Reports have shown that antidepressant drugs increase α_1-AR function (Holsboer and Barden, 1996). The α_1-AR have been implicated in the antidepressant-like responses of drugs in behavioural models of depression (Danysz et al., 1986, Kitada et al., 1983 and Masuda et al., 2001). These drugs increase the density of α_1-adrenoceptors and its agonist affinity (Deupree et al., 2007; Klimek et al., 1991). Previous study demonstrated that α_1-AR blockade in the central nervous system induced depression-related behaviour in the tail suspension test (Stone and Quartermain, 1999). In this study, SA2 shows non-specific binding affinity to the α_1-AR subtypes. The SA2 may offer new therapeutic option based on the fact that an indirect increase in adrenergic

transmission by some antidepressant drugs (reboxetine, desipramine, nortriptyline, maprotiline, and lofepramine) are characterised by adverse effects and not superior to SSRIs (Brunello et al., 2002).

Contrary to the proposed involvement of serotonergic mechanism in the antidepressant-like effect of SA2, the depletion of serotonin with PCPA did not attenuate effect of this compound. It was also hypothesized that SA2 could interact directly with serotonergic receptors especially 5-HT_{1A} receptor to elicit antidepressant-like effect. The presynaptic 5-HT_{1A} autoreceptors could inhibit serotonergic neuronal firing and release of vesicular serotonin. This receptor subtype is expressed postsynaptically in the hippocampus, hypothalamus, amygdala and cingulate and entorhinal cortices (Celada et al., 2013). The drugs activating 5-HT_{1A} receptors possess robust preclinical antidepressant like effects (Blier and de Montigny, 1987; Savitz et al., 2009). Agonist of 5-HT_{1A} such as buspirone has been shown to exert antidepressant-like effects and decrease the duration of immobility in the FST (Adell and Artigas, 1991). However, in this study, the antidepressant-like effect of SA2 was not blocked by WAY100635. These results exclude the role of 5-HT_{1A} receptors in the observed antidepressant-like effect of SA2. By implication, since classical hallucinogens are often a potent agonists of 5-HT_{1A} receptors (Nichols, 2004; David et al., 2014), non-blockade of SA2 antidepressant-like effect by WAY100635 suggests that an action of this compound could be devoid of 5-HT_{1A} receptors mediated hallucination.

Monoamine involvement in the antidepressant-like effect of SA2 was further evaluated through *in vitro* MAO assay. SA2 showed mild inhibition of MAO activity as compared to SA. However, SA2 is still far from being a potent inhibitor of MAO A and B as compared to Clorgyline and Deprenyl, respectively.

The unprecedented aspect of the present findings is associated with the antidepressant property of SA2 which showed high affinity for KOR and extremely low affinity SA2 for MOR and DOR. In general, agonist of KOR are known to induce pro- and antidepressant-like effects, respectively, in the forced swim and learned helplessness tests (Lutz and Kieffer, 2013). Although, SA2 showed preference for KOR, the binding affinity of this compound is still less as compared to that of salvinorin A. The KOR agonists including salvinorin A induce characteristic effects such as anhedonia, depressant-like and sedative-like behaviours in preclinical models (Butelman and Kreek, 2015). Although KOR antagonists can produce anti-depressant and anxiolytic-like effects (Carlezon et al., 2006; Rorick-Kehn et al., 2014), novel compounds with limited efficacy at KOR or compounds with "biased" KOR signaling, could induced antianxiety or antidepressant-like effect (Butelman et al., 2012b; Lovell et al., 2015).

The effect of SA2 possibly involves crosstalk among pathways and/or functional selectivity at KOR (biased agonism). Since dysphoria is a depressive like symptom, our results suggest that SA2 possesses antidysphoria property. In hypothesis, SA2 could possess bias KOR signaling through effector such as βarrestin2 rather than the receptor signals through G proteins. There are considerable evidences that KOR signals through βarrestin2 mediates sedation and dysphoria while through G proteins KOR signals mediate analgesic and antipruritic effects (Lovell et al., 2015). In hypothesis, SA2 could have antagonized βarrestin2-interacting receptor and suppressed βarrestin2 recruitment (through competition with dynorphin-stimulated βarrestin2 recruitment) among other downstream mechanisms. Since KOR modulation is a promising therapeutic target for the treatment of pain, drug addiction, and depression (Lovell et al., 2015), further studies of SA2 offer opportunity for new drug development in the future.

In summary, salvinorin A is a novel scaffold for the synthesis of new compounds with pharmacotherapeutic potential. Findings in this study demonstrated a potent antidepressant-like effect of SA2 in mouse models of depression without adverse effects on motor coordination. Although, our data are not sufficient to completely exclude hallucinogenic property that is associated with SA, it has been argued that the unique mind-manifesting properties of hallucinogens might offer synergistic effects to psychological care as compared with existing antidepressants (David et al., 2014).

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

Authors acknowledge supports from FAPEG, CAPES and CNPq.

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