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Investigation of *in vitro* Opioid Receptor Binding Activities of Some Turkish *Salvia* Species

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Abstract: Kappa Opioid Peptide Receptor (KOPr) activation produces analgesic, psychotomimetic, diuretic and antipruritic effects. KOPr ligands are investigated for their potential roles in the treatment of addiction, depression, feeding behavior, psychosis and schizophrenia. In this study the methanolic extracts of a number of *Salvia* species which are native to Turkey (*S. tomentosa*, *S. tchihatcheffii*, *S. rosifolia*, *S. dichroantha* and *S. sclarea*) were tested for their potential binding to opioid receptors in rat brain membranes and Chinese Hamster Ovary Cells expressing human KOPr (CHO-KOPh). [^{3}H]Diprenorphine, an unselective opioid antagonist, was utilized in the radioligand receptor binding assays. All extracts (0.11 mg/mL) inhibited the [^{3}H]Diprenorphine binding with ranging KOPr binding affinities. More than 50% inhibition of diprenorphine binding was shown only with *Salvia dichroantha* and *Salvia sclarea* both in rat brain membranes and CHO-KOPh membranes. Among them *Salvia sclarea* deserves further investigation for its active component(s) and its pharmacological characterization. This study clearly demonstrates the potential opioid receptor binding activities of several Turkish *Salvia* species. This work constitutes the first study on *in vitro* opioid receptor binding activities of *Salvia* species from the Turkish flora.

Keywords: Kappa opioid receptors; receptor binding activity; rat brain; radioligand binding; *Salvia.*

1. Introduction

Classical opioid pharmacology mainly arose from the isolation and characterization of alkaloids, such as morphine, from the opium poppy plant *Papaver somniferum*. Endogenous opioid peptides, the plant derived opioid alkaloids as well as their synthetic derivatives target the opioid receptors which are the important members of G-Protein Coupled Receptors. Four structurally similar

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but pharmacologically different opioid receptors have been discovered. Mu, Delta, Kappa and Nociceptin/Orphanin FQ peptide receptors are mainly localized in the central nervous system as well as the peripheral nervous system. The activation of the mu opioid receptor is mainly responsible for the analgesic and euphoric effects. Compounds acting on the mu opioid receptors have a limited use due to the tolerance-dependence to their effects, their abuse liability as well as the high risk of the respiratory depression [1]. Kappa opioid receptor activation is responsible for the analgesic and dysphoric effects. Kappa opioid receptor is recently investigated for its potential roles in the behavioral responses in substance abuse, depression, learning and memory [2].

In the 1960s, a novel member of the Lamiaceae family, *Salvia divinorum,* was discovered [3]. Traditionally, the leaves (by chewing or smoking) are used for its hallucinogenic effects for spiritual divination and also as a remedy for diarrhea, headache and rheumatism especially in Oaxaca, Mexico [4]. The isolation of the active component, salvinorin A, from the leaves of *Salvia divinorum* [5] started a new era in hallucinogenic compounds due to its unique non-nitrogenous chemical structure. Salvinorin A is the first non-nitrogenous diterpene that interacts very selectively with the KOPr and possesses psychotomimetic properties [2]. It is still legal to use *S. divinorum* extracts in many countries [6] and there is a large amount of work focusing on its pharmacological effects. Previously, in the tail flick assay, the antinociceptive property was shown to be mediated by the KOPr; also it has been shown that salvinorin A has a hypothermic effect and an anti-diarrheic effect mediated by the KOPr [7]. It has been postulated that Kappa Opioidergic System may have a role in mood disorders and there are reports indicating that salvinorin A produces depressive-like behavior [8]. On the other hand, one report exists for its use in refractory depression [9]. Sedation and loss of motor coordination produced by salvinorin A have also been shown [10]. Besides salvinorin A, several similar compounds (Salvinorin B-J) have been obtained from *S. divinorum* [11-14]. However, salvinorin A remains to be the most potent and effective, thus taken as a template compound for structure-activity studies for further synthesis of synthetic analogs [15,16].

Salvia (sage), the largest genus of the family Lamiaceae, consists of approximately 900 species which are distributed throughout the world [17]. The flora of Turkey is rich in *Salvia* species (commonly known as “adacayi”) and is represented by around 90 species, half of which are endemic [18]. Sage species are traditionally used as herbal teas in Turkey. Some of these species are spasmolytic, carminative, diuretic, antiseptic, and wound healing, as well as being used against colds [19]. In addition, aerial parts or leaves of several *Salvia* species are reported to be used to relieve various types of pain in Turkish traditional medicine, i.e., rheumatic pain, headache, toothache, abdominal pain, stomachache. Among these species the leaves of *S. tomentosa* are used to alleviate rheumatic pain [20] and abdominal pain [21], leaves of *S. dichroantha* are reported to reduce abdominal pain [22] and aerial parts of *S. sclarea* to relieve pain in sunstroke [23].

In this study, we aimed to investigate the possible opioid receptor binding activity of five *Salvia* species (*S. tomentosa* Miller, *S. dichroantha* Stapf., *S. sclarea* L. *S. tchihatcheffii* Boiss. and *S. rosifolia* Sm.) in rat brain membranes and cells expressing KOPr. To our knowledge, there is no *in vitro* receptor binding study performed on Turkish *Salvia* species as a first step for screening their opioid activity.

2. Materials and Methods

2.1. Plant Material

Salvia tomentosa, S. dichroantha (endemic) and *S. sclarea* were collected in July 2008 from Eskisehir, Turkey while *S. tchihatcheffii* (endemic) and *S. rosifolia* (endemic) were collected in July 2006 from Ankara and Bayburt, respectively. The plant materials were identified by Dr. Akaydin and the voucher specimens (Akaydın 10934, 10539, 11086, 11812 and 11028, respectively) have been deposited at the Herbarium of the Faculty of Education, Hacettepe University, Ankara, Turkey.

2.2 Chemicals

[^{3}H]Diprenorphine (specific activity 33 Ci/mmol) was purchased from Amersham GE HealthCare, UK. Diprenorphine and Salvinorin A are products of Tocris and Sigma-Aldrich, respectively. Tris-hydroxymethyl-aminomethane was purchased from Sigma-Aldrich. Methanol was provided from Merck Co., Darmstad. (-) Ethylketocyclozocine ((-)EKC), ((2alpha,6alpha,11S*)-3-(Cyclopropylmethyl)-6-ethyl-3,4,5,6-tetrahydro-8-hydroxy-11-methyl-2,6-methano-3-benzazocin-1(2H)-one) is a kind gift from Dr. Anna Borsodi (Hungary).

2.3. Preparation of the extracts

The air-dried and powdered leaves of each plant (1 g) were extracted with Methanol (10 mL) at 45 °C for 2 hours. After filtration, methanolic extracts were dried under vacuum. The extract yield was as follows: 15% for *S. tomentosa*, 13% for *S. dichroantha*, 15% *S. sclarea*, 16% for *S. tchihatcheffii* and 12% for *S. rosifolia*.

2.4. Animals

Inbred Wistar rats (Animal vivarium of the Yeditepe University, Istanbul, Turkey) were used throughout this study. Rats were kept four per cage, allowed free access to food and water and maintained on a 12/12-hour light/dark cycle until the time of sacrifice. Animals were treated according to the guidelines published in the European Communities Council Directives (86/609/ECC) and the Turkish Act for the Protection of Animals in Research (Regulation No. 25464) and the protocols were approved by the ethical committee of Yeditepe University (YUDETAM).

2.5. Membrane preparation

Wistar rats were decapitated and brains without cerebellum were quickly removed and washed several times with chilled 50 mM Tris-HCl buffer (pH 7.4), homogenized and filtered through four layers of gauze to remove large aggregates. The homogenate was centrifuged at 40,000 g (18,200 rpm) for 20 min at 4 °C. Pellet was re-suspended in fresh 50 mM Tris-HCl buffer and incubated for 30 min at 37 °C. The centrifugation step was repeated and the final pellets were resuspended in 50 mM Tris-HCl buffer (pH 7.4) and stored at -70°C.

Chinese Hamster Ovary cells stably expressing the wild type human Kappa Opioid Peptide receptor and Mu Opioid Peptide receptor (CHO-MOPh) were cultured in a medium containing Nut Mix F-12 (HAM) with L-glutamine (GIBCO Invitrogen) and 25 mM Hepes, 10 % FCS, 100 UI/mL penicillin, 100 µg/ml streptomycin and 0.4 mg/mL G418 at 37 ºC in a humidified atmosphere consisting of 5 % CO_2 and 95 % air. Cells were sub-cultured twice a week and harvested with ice cold phosphate Buffered Saline (PBS), frozen at -70 °C for 2 hours to facilitate cell disruption by water crystallization in 50 mM Tris-HCl (pH 7.4). Membrane was prepared as indicated above.

2.6. Competition Binding Assays

Aliquots of frozen membranes (60-80 µg protein/well) were thawed, and suspended in 50 mM Tris-HCl buffer (pH 7.4) and incubated with gentle shaking for 50 min, 24 °C in a final volume of 200 µl with the unlabelled compounds (10^{-5}-10^{-13} M) and/or extracts (0.11-0.00011 mg/ml) and ~1 nM [^{3}H]Diprenorphine (33 Ci/mmol, Amersham). The highest concentration of the extracts contains 1 % of methanol which has no interference with the binding parameters. Non-specific binding was determined in the presence of 10^{-5} M diprenorphine. The reaction was terminated by rapid filtration under vacuum (harvester 96 Mach III, TOMTEC) and washed with ice cold 50 mM Tris-HCl (pH 7.4)

buffer through GF/B glass fiber filters (Filtermat B 1450-521 Perkin Elmer). Filtermat was dried overnight and bound radioactivity was measured in an 8 mL Betaplate scint scintillation cocktail using a 1450 Microbeta Trilux Liquid Scintillation and Luminescence counter (Perkin Elmer). Protein concentration was measured by the Bradford method with bovine serum albumin as the standard [24].

2.7. Data Analysis

Data were analyzed with GraphPad Prism (version 5.0) by non-linear regression using the one-site competition fitting option. IC_{50} (the concentration of the competing ligand or extract at 50% inhibition of the specific binding) values were obtained from the displacement curves. Non-specific binding was subtracted from the total binding (in absence of ligand) and the specific binding was found. All receptor binding data are expressed as percentage inhibition of specific binding and are the means ± S.E.M. of the result of at least three independent experiments performed in duplicate.

3. Results and Discussion

Receptor binding experiments were performed with 0.8 ± 0.1 nM [^{3}H]Diprenorphine, a non-selective opioid receptor antagonist, on membranes from rat brain and cultured cells transfected with human KOPr. Reference compounds diprenorphine, ethylketocyclazocine (a full agonist of KOPr, pKi 10 [25]), and salvinorin A were also used to compete for the [^{3}H]Diprenorphine binding sites. Methanolic extracts prepared from the leaves of five *Salvia* species were able to displace the [^{3}H]Diprenorphine binding with variable affinities. IC_{50} values are summarized in Table 1a for compounds and 1b for the extracts. Heterologous displacement binding curves (Fig. 1) for each extract and salvinorin A were sufficiently fitted with the one-site binding model. In a brain membrane preparation a mixed population of receptors is present; however the recombinant systems are "purer systems" that carry only the desired receptor DNA and thus have only one type of receptor highly expressed. Therefore, in order to identify the specificity and selectivity of the opioid receptor binding, [^{3}H]Diprenorphine binding experiments were performed on membranes from Chinese Hamster Ovary cells expressing human KOPr (Fig. 1 C, 1 D). The two extracts from *S. dichroantha* and *S. sclarea* exhibited more than 50% binding to KOPr.

Table 1. The summary of the IC_{50} values obtained from the competition displacement binding curves for the ligands (**a**) and extracts (**b**). Inhibition (%) of the [^{3}H]Diprenorphine binding with the extracts (0.11 mg/mL) and ligands (10^{-5} M) are also shown. % displacement values are indicated with the S.E.M. (standard error of mean) values. (n.d.: not determined)

a)

Ligand	Rat Brain Membranes		CHO-KOPh Membranes	
	IC_{50} nM	**% inhibition at 10^{-5} M**	**IC_{50} nM**	**% inhibition at 10^{-5} M**
Diprenorphine	0.02	100 ± 0	0.26	100 ± 0
(-) Ethylketocyclozocine	n.d.	n.d.	0.35	99.4 ± 0.6
Salvinorin A	0.35	53.2 ± 6.7	5.1	87.9 ± 1.7

b)

Extract	Rat Brain Membranes		CHO-KOPh Membranes	
	IC_{50} mg/mL	**% inhibition at 0.11 mg/mL**	**IC_{50} mg/mL**	**% inhibition at 0.11 mg/mL**
S. tomentosa	0.01	37.1 ± 7.5	0.52	32.5± 18.5
S. tchihatcheffii	0.82	48.7 ± 4.6	0.21	40.0 ± 7.8
S. rosifolia	0.06	40.2 ± 5.6	0.06	57.3 ± 11.6
S. dichroantha	0.18	64.3 ± 2.9	0.001	45.2 ± 11.6
S. sclarea	0.27	60.2 ± 8.0	0.04	68.2 ± 2.4

The rank order of affinities and % inhibition is summarized in Table 1a and b. The affinity of salvinorin A in rat brain and CHO-KOPh membranes are in good agreement with the literature data [2]. Due to the extract compositions there can be many different compounds present; thus the affinities of the extracts are much lower when compared to the pure compounds. Mu Opioid Peptide receptor (MOPr) binding in CHO-MOPh cells were performed but the binding of the extracts was not detectable due to the shallow curves and thus the data are not shown. The problem with the shallow binding curves could not be overcome with increasing the extract concentrations possibly due to the higher organic solvent content which may cause membrane perturbation, which may affect the overall results.

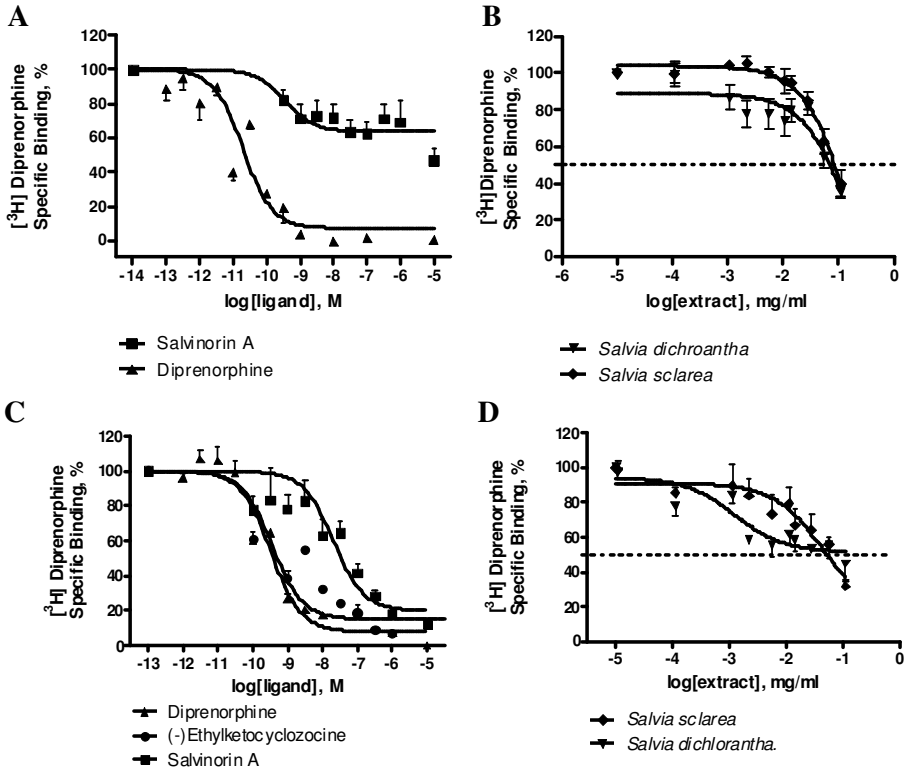

Figure 1. Displacement binding curves of salvinorin A, diprenorphine, *S. dichroantha, S. sclarea* in rat brain membranes (A, B) and in membranes from CHO-KOPh cells (C, D). [^{3}H]Diprenorphine (0.8 ± 0.1 nM) was incubated (50 min, 24 °C) in the presence of increasing concentrations of competitor ligands and extracts indicated in the figure. Points represent the mean ± S.E.M. of at least three different experiments each performed in duplicate.

The IC_{50} value of *S. tomentosa* is increased in CHO-KOPh membrane system. This may be due to the low capacity % inhibition of *S. tomentosa* at the highest concentration used in the experiment in the recombinant and cellular system with [^{3}H]Diprenorphine. Indeed the low IC_{50} (high affinity) values in rat brain membrane might indicate other targets than Kappa Opioid Peptide Receptors, since [^{3}H]Diprenorphine is a non-selective opioid receptor ligand. All of the other extracts have lower IC_{50} and higher % inhibition values that reflect the specificity of the binding towards kappa opioid peptide receptor. The order of specificity in terms of binding to the KOPr is *S. sclarea* > *S. rosifolia* > *S. dichroantha* > *S. tchihatcheffii* > *S. tomentosa.* The incomplete displacement binding curves observed with the extracts as well as Salvinorin A, both in rat brain and CHO-KOPh

membranes, may be due to i) possible allosteric binding to receptors, ii) the complex nature of the extracts with chemical diversity. In rat brain membranes the incomplete displacement binding curves may also be, in addition to the ones noted above, due to the possible non-specific interactions which may be a result of the non-selective opioid receptor antagonist ([^{3}H]Diprenorphine) that was used in this study.

In this paper, we have investigated the *in vitro* opioid receptor binding activity of crude methanolic extracts of five *Salvia* species, three of which are endemic to Turkey. Among these species, *S. sclarea, S. tomentosa* and *S. dichroantha* were selected based on their traditional utilization, including reported alleviation of various types of pain in Turkish folk medicine [21,22], while *S. tchihatcheffii* and *S. rosifolia* are endemic species not known for any healing features. Previously Salvinorin A was shown to inhibit only partially the [^{3}H]Diprenorphine binding [27]. This study has clearly demonstrated that these two species may produce their pain-relieving effect at least to some extent through opioid receptors.

Previous phytochemical studies on *Salvia* species indicated the presence of diterpenes and phenolic compounds as the chief secondary metabolites [17]. The pharmacological activities of the *Salvia* species are mostly attributed to their diterpene contents, as in the case of *S. divinorum* (salvinorin A) and *S. miltiorrhiza* (tanshinones) [28]. Eventually, the opioid receptor binding activities of the extracts may arise from the diterpenoid component of these species. The most active species, *S. sclarea* and *S. dichroantha* need further investigation for the identification of the active components responsible for this activity. There are only a few studies in the literature that were performed on several *Salvia* species; Li and colleagues observed no significant binding to any of the opioid receptors with the compounds isolated from *S. splendes* (splendindin) and *S. farinacea* (salvifaricin) [29]. Moreover, *S. leriifolia* and *S. miltiorrhizae* have been shown to possess antinociceptive effects claimed to be mediated by opioid receptors [30, 31]. The pharmacological effects of *Salvia* species on the central nervous system are summarized in a review [28].

Morphine is one of the classical examples of the use of natural plant sources as the pharmacologically active remedies for many diseases. It is a partial agonist acting on the mu opioid receptor and the most well known analgesic drug in the world. Almost all the drugs acting on the opioid receptors have a morphinan structure (with nitrogenous heterocyclic group). This structure is conserved as the distance between the aromatic group and the nitrogen even in the opioid peptides and the presence of the positively charged nitrogen atom was an assumed requirement for the interaction with opioid receptors [32]. Due to the conserved structure of the opiates, besides the analgesic effect, the undesirable side effects like tolerance and dependence and abuse potential emerge, thus their usage is limited and the undesired side effects are believed to be related to the classical morphinan structure. In 1994, a neocleredane diterpene (salvinorin A), the first non-nitrogenous hallucinogenic compound, was isolated from *S. divinorum* and can bind with high affinity to KOPr [5]. The Kappa Opioidergic system is mainly associated with hallucinations in mood disorders or schizophrenia and Alzheimer's disease. Salvinorin A is also an allosteric modulator of the MOPr [27]. Allosteric modulators are becoming more and more popular for their potential in drug development [33]. Salvinorin A is one of the few allosteric modulators of opioid receptors; therefore its structure deserves attention for templates of novel ones. The non-classical diterpenoid structure with the allosteric modulator capability of salvinorin A and similar compounds that are isolated from other *Salvia* species, can be lead structures for novel ligands acting on mainly opioid receptors. As stated by Christopoulos (2002) [26], in contrast to orthosteric ligands, an allosteric compound may have no apparent effect on orthosteric radioligand binding even when it showed prominent allosteric modulation on the signaling. Moreover in a plant extract there are no simple pure compounds but rather those with more complex chemical structures. Therefore our results may not rule out the possibility of diterpenoid content and the allosteric capability of the extracts to the Mu Opioid Receptor. It is well known that diterpenes are common in the genus *Salvia* [34, 35]. The structural diversity of the diterpenes within the genus *Salvia* varies greatly. So, the structures of the diterpenes which could be present in the non-active extracts might be different from those of *S. sclarea* and *S. dichroantha*. Although several triterpenoids and diterpenoids have been reported from the roots of *S. dichroantha* [35] and *S. sclarea* [36], their aerial parts have been less investigated. If a greater number of novel compounds from different *Salvia*

species are identified, more receptor specific interactions and better ligands can be designed. Therefore it would be intriguing to perform structural elucidation of the active components in these *Salvia* species, in particular the *Salvia sclarea*.

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