5-HT_{2A} RECEPTOR BINDING AND ANTIDEPRESSANT STUDIES ON ANXIMIN®, A POLYHERBAL FORMULATION

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Summary

The objective of the present study was to carry out preclinical evaluation of the antidepressant activity of a polyherbal formulation Anximin. It consists of 5 medicinal plants namely: *(i) Bacopa monnierae, (ii) Convolvulus pluricaulis, (iii) Rauwolfia serpentina, (iv) Nardostachys jatamansi and (v) Acorus calamus.* Adult Charles foster albino rats (200±20g) and Wistar mice (25±5g), of either sex were used for the study. Antidepressant activity was assessed by using the validated models of depression viz. behavioural despair test (BDT), tail suspension test (TST), learned helplessness test (LHT) and yohimbine toxicity enhancement test (YTE). Since single acute administration of both the doses of Anximin (20 and 40 mg/kg) suspended in 0.3% CMC had no significant antidepressant effect, therefore it was given orally for 7 consecutive days. The standard drug imipramine (10 mg/kg) was also administered for 7 consecutive days through oral route. All the behavioural experiments were performed 1 hr after last administration of drug on day 7. Immobility time in FST and TST was significantly ($p < 0.01$) reduced by the high dose (40 mg/kg) of Anximin treated animals, while low dose (20 mg/kg) has no significant effect. A significant ($p < 0.001$) decrease in number of escape failures in LHT was also observed in both groups of Anximin treated rats. In YTE test, the percentage of mortality of animals was 33% and 66% with both the doses (20 and 40 mg/kg respectively) of Anximin whereas in imipramine treated group mortality was 100%. To elucidate mechanism of action, a receptor binding study was also performed using rat's frontal cortex. The high dose (40 mg/kg) of Anximin treated group significantly ($p < 0.05$) decreased the binding level of ^{3}[H] ketanserin, indicating a downregulation of 5-HT_{2A} receptors. The results indicate that Anximin possesses promising antidepressant activity acting through 5-HT_{2A} receptors.

Key words: Anximin; Antidepressant; Receptor binding; 5-HT_{2A} receptors.

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Introduction

Depression is a serious clinical condition of emotional dysregulation (depressed mood, irritability, anxiety) accompanied by severe disturbances of fundamental physiological systems. Clinical depression is currently the leading cause of disability and, according to the World Health Organization, is expected to become the second leading cause of disease-related disability by the year 2020, following heart disease [1]. Depression is the result of several causes but psycho emotional and psychosocial stresses associated with crisis and conflict life events represent the most common pathogenic factors resulting in depression. The level of stressful and traumatic events is dramatically increased nowadays worldwide which extends the risk of stress-related depressive pathologies, and the frequency of clinical forms of depression is constantly escalating. Hence, a search for new effective methods of prophylaxis and treatment of human depression, as well as clarifying the adaptive brain mechanisms contributing to these processes, represents one of the most pressing problems facing modern neurobiology and medicine.

Currently available therapy for depression treatment is often associated with several undesirable side effects, and it is effective only in a certain portion of the population [2, 3]. Therefore, the identification of alternative therapeutic tools for the treatment of depression is still needed. Herbal therapies may be effective alternatives in the treatment of depression, as in the case of St John's wort [4-6], and the search for novel pharmacotherapy from medicinal plants for psychiatric illnesses, including depression, has progressed significantly in the past decade [7]. It is interesting to note that most of the novel treatments for depression (including St. John's wort) seem to act through a mechanism which does not differ significantly with respect to that of "classical" antidepressants. In Ayurveda, compound formulations are generally used in therapy, based on the premise that such a combination would provide a synergistic therapeutic effect and help to minimize the adverse effects of the major drugs [8]. Anximin is one such herbal formulation containing five Indian medicinal plants: *Bacopa monnierae (BM), Convolvulus pluricaulis (CP), Rauwolfia serpentina, Nardostachys jatamansi (NJ) and Acorus calamus (AC).* Some of these plants have been reported to have antidepressant activity. BM extract or bacosides have shown antidepressant activity [9]. Some studies have shown that shankhpushpi has potent depressive action in mice [10]. Results from behavioural tests have also revealed that an extract from NJ exhibited significant antidepressant activity [11].

Materials and Methods

Animals: The experiments were performed on adult Charles Foster albino rats (200 ± 20g) and Wistar mice (25 ± 5g), of either sex, procured from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University; and were randomly distributed into four groups of six animals each. The rodents were housed in groups of six in polypropylene cages at an ambient temperature of 25^0C ± 1^0C and 45-55% RH, with a 12:12 hr light/dark cycle. Animals were provided with commercial food pellets and water *ad libitum* unless stated otherwise. Experiments were conducted between 09.00 and 14.00 hr. Animals were acclimatized for at least one week before using them for experiments and exposed only once to every experiment.

Drug administration: Animals were randomly divided into four groups, each consisting of 6 animals, and Anximin was administered orally as 0.3% carboxymethyl cellulose (CMC) suspension, in the doses of 20 and 40 mg/kg, once daily for seven consecutive days. Control animals were treated with equal volume of vehicle (0.3% CMC suspension). Imipramine (10

mg/kg), standard antidepressant agent, was administered orally to rats for seven days [11]. Experiments were conducted on day 7, 1hr after the last drug administration.

Methods:

1. *Behavioural despair test (BDT):* The method of Willner, 1984, was followed [12]. The rat was placed in a cylinder (45 × 20cm) containing 38 cm water (25 ± 2^0 c), so that the rat could not touch the bottom of the cylinder with its hind limb or tail, or climb over the edge of the chamber. Two swim sessions were conducted, an initial 15 min pre-test, followed by a 5 min test 24 h later. Drugs were administered after pre-test. The period of immobility (remained floating in water without struggling and making only those movements necessary to keep its head above water) during 5 min test period was noted.

2. *Tail suspension test (TST):* The method of Chermat, et al., 1986, was followed [13]. A mouse was hung on a wire in an upside down posture so that its nostrils just touch the water surface in a container. After initial vigorous movements, the mouse assumes an immobile posture and the period of immobility during a 5-min observation period were noted. This test is a reliable and rapid screening method for antidepressants, including those involving the serotonergic system [14].

3. *Learned helplessness test (LHT):* The method of Sherman, et al., 1982, was followed [15].
(a) Inescapable shock treatment: Rats were subjected to foot shocks in a two compartment jumping box with the escapable route to the adjoining unelectrified 'safe' chamber closed. A constant current shocker was used to deliver 60 scrambled shocks (15 sec duration, 0.8 mA every min) through the steel mesh grid floor. Control animals were placed in the chamber for 1 hr without experiencing shocks. This exercise was repeated 48 hr later on day 3.

(b) Conditioned avoidance training: In order to evaluate escape and avoidance performance, avoiding training was initiated 48 hr after inescapable shock pre-treatment in the Sidman jumping box (Techno, Lucknow, India). The jumping box was divided into two equal chambers (27 x 29 x 25 cm) by a plexy glass partition with a gate providing access to the adjacent compartment through a 14 x 17 cm space. Animals were placed singly in one of the chambers of jumping box and were allowed to habituate to the test environment for 5 min. (for the first session only) and then were subjected to 30 avoidance trails (inter-trial intervals being 30 s). During the first 3 s of each trial, a light signal was presented, allowing the animals to avoid shocks. If a response does not occur within this period, a 0.8 mA shock (3 s duration) was applied via the grid floor. In case no escape response occurs within this period, shock and "light conditioned stimulus" were terminated. Avoidance sessions performed for 3 consecutive days (day 3, 4 and 5) in the morning, and the number of escape failures, referred as no crossing response during shock delivery was recorded [9].This model has excellent predictive validity and is extensively used to screen antidepressants [12].

4. *Yohimbine toxicity enhancement (YTE):* The method given in Vogel, et al., 2002, was followed [17]. Groups of 10 male mice (25–28 g) were used. Mice were placed in plastic cages and receive the test compound or the vehicle by oral or i.p. administration. Thirty min later, a dose of 25 mg/kg yohimbine (a sub lethal dose) was given subcutaneously. Mortality rate was assessed 1 hr, 2 hr, 4 hr, and 24 hr after dosing. Lethality in the control group (yohimbine only) is less than 10%, whereas 10 mg/kg imipramine-HCl causes death in about 90%.

5. *5-HT2A receptor binding assay:* All the animals were sacrificed by means of quick decapitation after 1 hr of last drug administration. Brain was dissected quickly and whole of frontal cortex was isolated as described by Glowinski and Iversen, 1966 [18]. The crude synaptic membranes were prepared as described earlier by Khanna, et al. 1994 [19]. Briefly, brain regions were weighed and 20% homogenates were prepared using an Ulraturix and adding 19 volumes of 5 mM Tris buffer (pH 7.4). The homogenate was centrifuged at 50,000 × *g* for 10 min at 4 C. Subsequently, the pellet was washed with buffer and was rehomogenised and again centrifuged at the same speed for 10 min to remove endogenous amines. Finally, the pellet was suspended in Tris–HCl buffer (40 mM, pH 7.4) and stored at -80 C till receptor binding assay [20].

Binding incubations were carried out in triplicate in a final volume of 1ml containing 40mM Tris–HCl buffer, pH 7.4, and the appropriate labelled and unlabeled pharmacological agents. The amount of tissue used per tube corresponded to 5–10 mg of the original wet weight and contained 250–300 µg of membrane protein as determined by the method of Lowry, et al. 1951 [21]. All additions were made at 4 C in ice bath. At the end of 15 min incubation at 37°C; samples were filtered under vacuum through glass fiber discs (GF/B, 25mm diameter, 0.3 µm pore sizes, Whatman, USA). The filters were washed twice with 5ml of cold Tris–HCl buffer (40 mM, pH7.4) to remove unbound radioligand. The filter discs were then dried and counted in 5 ml of scintillation fluid (cocktail-containing: 2,5-Diphenyl oxazole (PPO), 1,4, bis 5-Phenyl oxazolyl benzene (POPOP), naphthalene, toluene, methanol and dioxin) using a beta scintillation counter (Packard Instruments Co., USA) at an efficiency of 40-50 % tritium, to determine membrane bound radioactivity.

Control incubations, containing unlabelled competing ligand, were carried out simultaneously with the experimental series to determine the extent of nonspecific binding. The final concentration of unlabelled competing compounds in the control incubations was 1×10^{-5} M in ethanol. Specific binding was taken to be the binding that was displaced in the presence of this large excess of the competing compound and was calculated as total binding minus the nonspecific binding. The assay for 5-HT_{2A} receptor was performed by using 1.5×10^{-9} M ^{3}[H] ketanserin (76.50 Ci/mmol) as the binding ligand and unlabelled cinanserin (1×10^{-5} M) as the competing compound in the control tubes. The method used was essentially similar to other filtration binding methods Yamamura, et al., 1978 [22] and satisfies the requirements for saturability, specificity, reversibility and regional distribution [23, 24]. The values presented are representative of three separate runs, each in triplicate, performed on the pooled samples of six animals in each series. All individual values of control and experimental groups were found to be within the 95 % confidence limits and the values were expressed as pmol bound/g protein.

Statistical Analysis: All values were expressed as mean ± SEM. Statistical significance between control and treatment groups was analysed by one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test. Statistical significance between same treatment groups was analysed by Student's t-test. Statistical significance was determined at 5% level of confidence ($p < 0.05$). GraphPad InStat (version 3.06) software was used for all statistical analysis.

Results

BDT: In the initial experiments acute administration of even high doses of Anximin (40 mg/kg) did not reveal any antidepressant like effects in this test. Therefore repeated oral administration of 20 and 40 mg/kg of Anximin for seven consecutive days was done. Only the high dose group significantly ($p < 0.01$) reduced the immobility time in rats. Standard drug imipramine showed similar activity and its effects were qualitatively comparable to the high dose of Anximin (40 mg/kg). The observed effects of the Anximin are summarised in Table: 1.

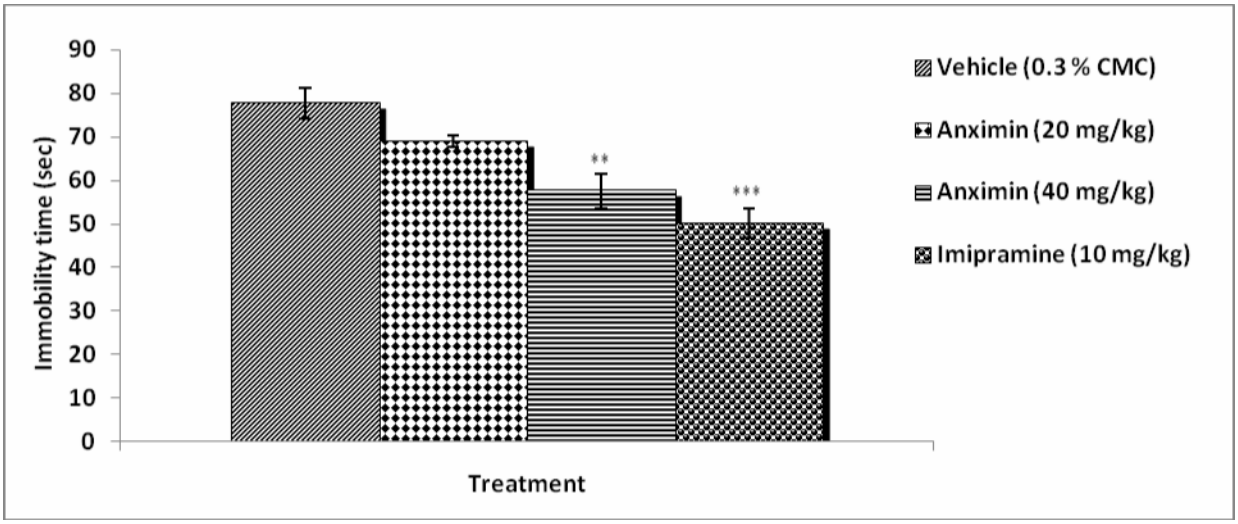
Table 1: Effect of Anximin on behavioural despair test in rats

Treatment	Immobility time (sec)
Vehicle (0.3 % CMC)	109.12 ± 7.54
Anximin (20 mg/kg)	96.51 ± 3.35
Anximin (40 mg/kg)	81.08 ± 2.67**
Imipramine (10 mg/kg)	73.38 ± 3.61***

Values are mean ± S.E.M., n= 6. Asterisks denote statistically significant differences relative to vehicle (** =$p < 0.01$ and *** =$p < 0.001$)

TST: Anximin caused significant ($p < 0.01$) decrease of the immobility time in TST at a higher dose (40 mg/kg). However, the lower dose (20 mg/kg), of Anximin treated group did not show any significant reduction in immobility time. Imipramine showed significant ($p < 0.001$) antidepressant activity and its effects were qualitatively comparable to the high dose of Anximin Fig: 1.

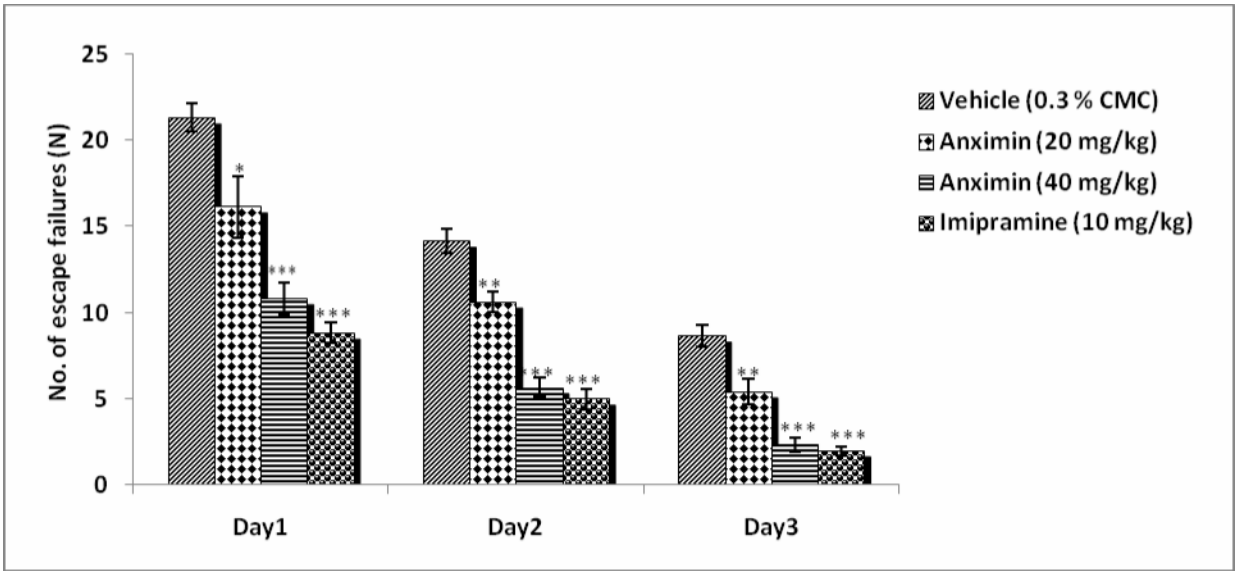
Fig 1: Effect of Anximin in tail suspension test in mice



Values are mean ± S.E.M., n= 6. Asterisks denote statistically significant differences relative to vehicle (** =$p < 0.01$ and *** =$p < 0.001$)

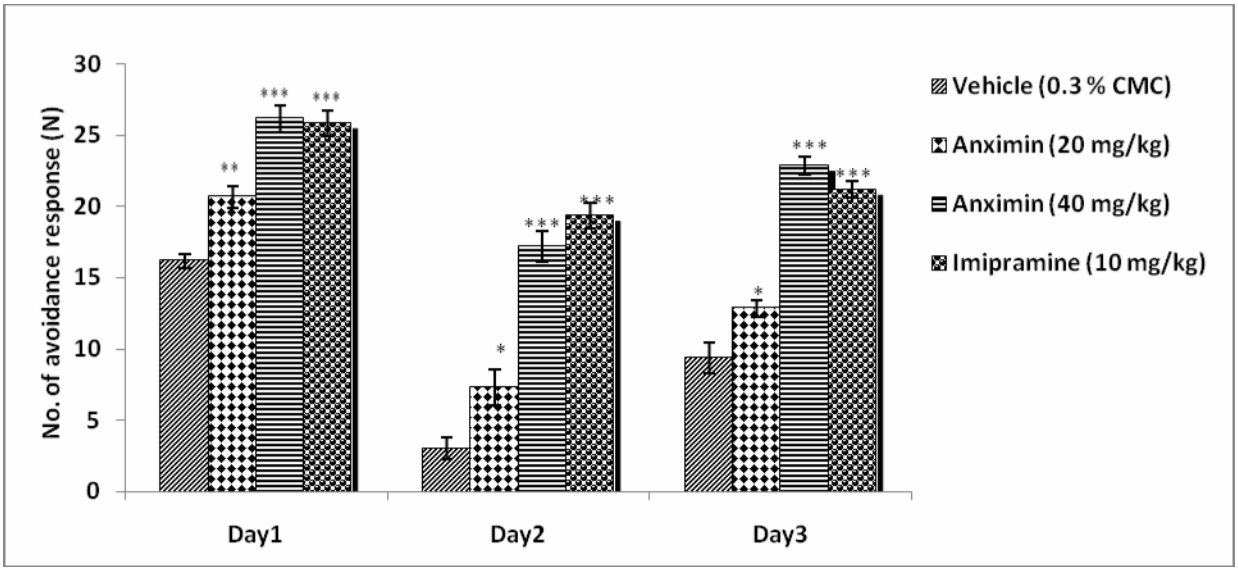
LHT: Vehicle treated rats with prior experiences of inescapable shocks exhibited marked increase in escape failures as compared to those with no such prior experiences. The escape failures significantly decreased ($p < 0.01$ and $p < 0.001$) in rats treated with both the doses of Anximin respectively. Additionally the number of avoidance response also significantly increased ($p < 0.05$ and $p < 0.01$) respectively, for both the doses of Anximin. Imipramine also showed a significant ($p < 0.001$) reduction of learned helplessness and its effects were qualitatively comparable to that of Anximin. The results are shown in Fig: 2 and 3.

Fig 2: Effect of Anximin in learned helplessness test in rats



Values are mean ± S.E.M., n= 6. Asterisks denote statistically significant differences relative to vehicle (* =$p < 0.05$, ** =$p < 0.01$ and *** =$p < 0.001$)

Fig 3: Effect of Anximin in learned helplessness test in rats



Values are mean ± S.E.M., n= 6. Asterisks denote statistically significant differences relative to vehicle (* =$p < 0.05$, ** =$p < 0.01$ and *** =$p < 0.001$)

YTE: The simultaneous administration of Anximin and yohimbine lead to death of animals, due to an increased concentration of nor-adrenaline. The lethality expressed as percentage was 33% and 66% at 20 and 40 mg/kg doses of Anximin respectively. Administration of 10 mg/kg of imipramine leads to a 100% lethality. Results are summarised in the Table: 2.

Table 2: Effect of Anximin on Yohimbine toxicity enhancement test in mice

	LETHALITY					
Treatment	**1 hr**	**2 hr**	**4 hr**	**24 hr**	**Total**	**Percentage (%)**
Vehicle (0.3 % CMC)	0	0	0	0	0	0
Anximin (20 mg/kg)	0	1	1	0	2	3335
Anximin (40 mg/kg)	1	1	1	1	4	66.66
Imipramine (10 mg/kg)	2	2	1	1	6	100

5-HT$_{2A}$ receptor binding assay: Specific binding of ^{3}[H] ketanserin in frontal cortex of control rats was 122.3 ± 5.68 pmol/g protein. Anximin (40 mg/kg) significantly ($p < 0.05$) decreased the binding level of ^{3}[H] ketanserin to 97 ± 3.66 pmol/g protein in frontal cortex indicating the down regulation (-21%) of receptor. However, no significant alteration in the binding level of ^{3}[H] ketanserin (112 ± 9.39 pmol/ g protein) was observed in Anximin (20 mg/Kg) pre-treated rats (Table 3).

Table 3: Effect of Anximin on high affinity 5-HT $_{2A}$ receptors in frontal cortex of rats

Brain region	**Receptor Ligand**	**Treatment**	**pmol bound/g protein**	**% change**
Frontal cortex	5-HT ^{2A} 3[H] ketanserin	Vehicle (0.3 % CMC)	122.30 ± 5.68	---
		Anximin (20 mg/kg)	112.00 ± 9.39	8.42
		Anximin (40 mg/kg)	97.00 ± 3.66*	20.68

Values are mean ± S.E.M., n= 6. Asterisk denotes statistically significant differences relative to vehicle (* =$p < 0.05$)

Discussion

Amongst a wide variety of proposed and critically assessed *in vivo* models of depression [13], [25], two most commonly used paradigms are learned helplessness [26, 27] and forced swimming tests [28]. Behavioural despair test was proposed as a model to test for antidepressant activity by Porsolt, et al., 1978 [29]. It was suggested that mice or rats forced to swim in a restricted space from which they cannot escape, exhibit a characteristic immobility [30-33]. The behaviour reflects a state of despair, which can be reduced by several agents, which are therapeutically effective in human depression [34]. In LHT, rodents are exposed to inescapable and unavoidable electric shock in one situation, later fail to escape shock in a different situation when escape is possible [35, 36]. This phenomenon was evaluated as a potential animal model of depression [16].

Apart from these two paradigms, the observed results in TST and YTE provide additional measures for assessing antidepressant activity. In TST, the immobility displayed by rodents when subjected to an unavoidable and inescapable stress has been hypothesised to reflect depressive disorders in humans. Clinically effective antidepressants reduce the immobility that mice display after active and unsuccessful attempts to escape when suspended by tail [34].

In YTE, yohimbine occupies central α2-receptors, and prevents nor-adrenaline from binding to these receptors. Compounds with antidepressant properties are known to inhibit physiological inactivation of nor-adrenaline and other biogenic amines by blocking the re-uptake at nerve terminals. Administration of a test compound with antidepressant properties leads to an increase in nor-adrenaline concentration. Following the simultaneous administration of yohimbine and antidepressants the animals die of nor-adrenaline poisoning. The test has been proven as a simple method to detect antidepressants with monoamine uptake inhibiting properties [17].

Depressive disorder has long been associated with disturbances of brain serotonin (5-hydroxytryptamine [5-HT]) activity and data concerning 5-HT variations in depression have probably been the most widely studied. Moreover, the serotonergic system plays a major role in the action of antidepressants [37]. Tremendous evidence supports that dysfunction of serotonergic neurotransmission is implicated in the pathogenesis of mood disorders including depression and anxiety [38-42]. One of the main target structures of the serotonergic system is prefrontal cortex (PFC), a brain region highly associated with the control of emotion and cognition [43]. Specific changes of the PFC serotonin system found in patients with mental disorders [42], [44, 45] suggest that serotonin plays a crucial and unique role in PFC.

Although 5-HT has been implicated in the pathophysiology of depression, the precise nature of alterations in the 5-HT system that underlie depressive symptoms still remains elusive. The 5-HT acts on at least 14 subtypes of 5-HT receptors (5-HT_1 to 5-HT_7 subfamilies), and, of these, 5-HT_2 receptors have been the most studied in suicide victims with or without a history of depression and in depressed patients who died of natural causes. Most, [46-49] although not all, [50, 51] post-mortem studies in suicide victims with mixed, uncertain, or no psychiatric diagnosis reported an increase in brain 5-HT_2 receptor binding, particularly in frontal cortex, compared with control subjects. For instance, $5HT_{2A}$ receptor down-regulation is a common mechanism of antidepressant action, and increased densities of $5HT_{2A}$ receptors have been reported in the brains of depressed patients [47], [52]. Their therapeutic effects, however, are not immediate and appear only after administration of the compounds for

approximately two weeks [53]. This delay is postulated to be due to down-regulation of noradrenergic and serotonergic receptors [54, 55].

The present finding of the receptor radioligand binding studies showed that high dose of Anximin (40 mg/kg) has significantly decreased the binding of the [^{3}H] ketanserin in the frontal cortex. This indicates a downregulation of 5-HT_{2A} receptors. This mechanism may be responsible for the observed antidepressant activity of Anximin.

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