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EVALUVATION OF THE EFFECT OF NICOTINIC RECEPTOR ANTAGONIST WITH ANTIDEPRESSENTS IN MICE

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ABSTRACT

Objectives: Major depression is a heterogeneous disorder it could not be fully controlled by a drug possessing one discrete mechanism of action so multi-modal treatment concepts are attracting attention. Nicotinic receptor antagonist had role in the treatment of depression. Therefore, this study was carried out to investigate the possibility of synergistic potential of dextromethorphan which is a nicotinic receptor antagonist with antidepressants for the treatment of depression. Also the effect of this combination on pain is tested in this study. **Methods and Materials:** Antidepressants such as duloxetine and venlafaxine at their median effective dose that is 30mg/kg, 8mg/kg i.p. respectively, were evaluated in combination with dextromethorphan 30mg/kg intraperitoneally for the synergistic potential for ameliorating depression in Swiss albino mice. Behavioural studies are carried out using forced swim test and tail suspension test. This was followed by the locomotor activity. The effect of these drugs on pain was checked by Eddy's hot plate and tail immersion methods. The seratonin expression in brain was studied by RT-PCR method. **Results:** Behavioral studies indicated that antidepressant venlafaxine and duloxetine with dextromethorphan resulted in significant reduction in immobility time compared to the vehicle control in FST and TST, respectively. All the data were evaluated using the one-way analysis of variance followed by individual comparisons using Tukey's *post-hoc* test. Locomotor activity studies demonstrated no significant increase in general locomotion after co-administration of the compounds which shows that the reduction in duration of immobility with these drugs was due to antidepressant effect. RT-PCR shows more receptor expression in combination group. **Conclusion:** The present results suggest the concoction of dextromethorphan with venlafaxine and duloxetine for enhanced synergistic antidepressive effects with the reduction of dose.

KEYWORDS: Antidepressants, Depression, Dextromethorphan, Duloxetine, Eddy's hot plate, Forced swim test, Nicotinic receptor antagonist, Tail immersion test, Tail suspension test, venlafaxine.

INTRODUCTION

Depression is a serious disorder which, according to the World Health Organization, is one of the most prevalent and expensive psychiatric disorders of the developed world with a lifetime prevalence of 7.5% and 17%. Moreover1 it is a mood disorder that affects the minds as well as the body described as a state of feeling extremely low, to the extent that it affects a person's thoughts, behavior, feelings, and general well-being. While all of us have temporary periods during which we feel unhappy, depression isn't sometimes a person just snaps out of or gets over .It's more long term; it needs a thorough diagnosis and treatment involving counseling and medication; and above all, it's not a weakness that a person has, but rather a medical condition like any other [1]. Depression is characterized by a number of common symptoms. These include a persistent sad, anxious, or "empty" mood, and feelings of hopelessness or pessimism. A person who is depressed also often has feelings of guilt, worthlessness, and helplessness. They no longer take interest or pleasure in hobbies and activities that were once enjoyed; this may include things like going out with friends or even sex. Insomnia, early-morning awakening, and oversleeping are all common.[2]

Antidepressants,[3] are the most prescribed therapy for depression. The exact mechanism of action of antidepressants is unknown. The prevailing theory is that antidepressants increase the concentration of one or more brain chemicals (neurotransmitters) that nerves in the brain use to communicate with one another. The neurotransmitters affected by antidepressants are norepinephrine, serotonin, and dopamine. The different classes of antidepressants differ in the neurotransmitters they affect. This determines some of their side effects and potential drug interactions. All available

antidepressants are effective, and for most cases of depression there is no good evidence that any antidepressant is more effective than another. Side effects and potential drug interactions are major factors that influence selection of antidepressants and compliance with therapy. Selective serotonin reuptake inhibitors were developed more recently than TCAs and are the most widely used class of antidepressants. They work by increasing the level of serotonin in the brain. Unlike MAOIs and TCAs, they do not significantly affect norepinephrine levels in the brain. SSRIs also have fewer and milder side effects, fewer drug interactions, and are much less likely to be associated with suicide than TCAs. Serotonin norepinephrine reuptake inhibitors (SNRIs) are the newest class of antidepressants. SNRIs work by increasing the levels of serotonin and norepinephrine that are active in the brain. Serotonin and norepinephrine are produced by nerves and released into the surrounding tissues where they can attach to nearby receptors on other nerves, thereby stimulating the other nerves. The released serotonin and norepinephrine then are taken up and released again by the nerves that produce them. SNRIs block the uptake ("reuptake") of the serotonin and norepinephrine so that more of the serotonin and norepinephrine are free in the tissues surrounding the nerves.[4]

Neuronal nicotinic ACh receptors (nAChRs) belong to the family of ligand-gated channels. These receptors constitute both the ligand-binding site and the ionic pore through which ions can flow when the receptor is stabilized in the open conformation. Historically, the existence of such receptors was first revealed in 185715 by Bernard, who showed that the poison curare blocks transmission at the neuromuscular junction, but does not prevent muscle contraction elicited by electrical stimulation. Since this observation, the neuromuscular junction has been used as a reference for synaptic transmission in physiology and pharmacology. It was also recognized a long time ago that ACh is the neurotransmitter that acts on the parasympathetic ganglia, but little was known about the precise mechanisms underlying this neurotransmission [5]. One of the most consistent findings in neuropsychiatry is that patients with depression have dysfunctional neuroendocrine systems possibly resulting from prolonged responses to stress. The available evidence suggests that acetylcholine (ACh) plays a significant role in mediating neuroendocrine, emotional, and physiological responses to stress. For example, central acetylcholine turnover is increased following stress and Ach facilitates the release of several stress-sensitive neurohormones and peptides including corticosterone, ACTH, and CRF. The cholinergic-adrenergic theory of depression hypothesizes a balance between cholinergic and adrenergic systems, suggesting that over activity of the cholinergic system over the adrenergic system could lead to depressive symptoms.[6] Consistent with this hypothesis, strong evidence supports the presence of exaggerated responses (behavioral, neurochemical,

sleep) to cholinergic agents in affective disorder patients relative to controls.[6] For example, the indirect ACh agonist, physostigmine, when administered to normal subjects, causes an increase in heart rate and blood pressure and produces symptoms of dysphoria, depression, anxiety, irritability, aggressiveness and hostility.[7]

Nicotinic antagonists.[7] inhibit the effects of acetylcholine on nicotinic receptors. According to their dominant effects, we distinguish the antagonists acting on the autonomic nervous system which are called ganglionic blocking agents, and those acting on neuromuscular junction which are called neuromuscular blocking agents. A nicotinic antagonist is a type of anticholinergic drug that inhibits the action of acetylcholine (ACh) at nicotinic acetylcholine receptors. These compounds are mainly used for peripheral muscle paralysis in surgery, the classical agent of this type being tubocurarine,[8] but some centrally acting compounds such as bupropion, mecamylamine, and 18-methoxycoronaridine block nicotinic acetylcholine receptors in the brain and have been proposed for treating drug addiction. The nicotinic receptors of autonomic ganglia and skeletal muscle are not identical; they respond differently to certain stimulating and blocking agents, and their pentameric structures contain different combinations of homologous subunits. Trimethaphan and hexamethonium are relatively selective competitive and noncompetitive ganglionic blocking agents.[9] Although tubocurarine effectively blocks transmission at both motor end plates and autonomic ganglia, its action at the former site predominates. Succinylcholine, a depolarizing agent, produces selective neuromuscular blockade.[10]

MATERIALS AND METHODS

Normal saline
0.9 % w/v sodiumchloride solution was used for the study. Normal saline is the commonly used phrase for a solution of 0.90% w/v of NaCl, 308 mOsm/L or 9.0 g per liter. At first 9gm of corium chloride (Nacl) are weighted with balance. 600ml of distilled water is taken in a beaker/volumetric flask and added 9gm Nacl. Mixed it properly then distilled water is added up to 1000 ml again mixed it with the help of stirrer.

Drugs
The drugs used in studies such as duloxetine, venelafaxine and dextromethorphan were purchased from Yarrow chem products, mumbai in the pure form. ED50 doses of two antidepressants were fixed according to previous research work done on its26. ED50 doses were then used in combination with dextromethorphan to study for synergistic potential. The dose of dextrometorphan 15 mg/kg i.p was selected based on previous research work done on dextrometorphan. Animals were randomized on the basis of their body weight into different groups such as vehicle p.o. (Group 1), Venlafaxine 8mg/kg (Group 2), Duloxetine 30mg/kg

(Group 3) Dextromethorphan (DXM) 30mg/kg (Group 4), Venlafaxine 4mg/kg+ DXM15mg/kg (Group 5), Duloxetine 15mg/kg+DXM15mg/kg (Group 6).

Animals
Swiss albino mice of either sex weighing 25-30g were used for this study. The animals were purchased from Sree chithra Tirunal Institute of Medical Science and Technology, poojapura, Trivandrum. Those animals were housed in the groups of 6 mice/cage in standard cages in a room temperature of $22 \pm 2°C$, under natural light/dark cycle and had free access to water and food (standard laboratory pellets) before the experiments. The mice were acclimatized at lab conditions for 5 days before the start of the experiment. All the experimental work had been carried out from 9:00 to 16:00. All experimental pharmacologic studies were done after getting permission from the Institutional Animal Ethics Committee, Ezhuthachan College of Pharmaceutical Sciences, Marayamuttom (2/IAEC/Pharmacology/ECP S/2015) and care of animals was taken as per CPCSEA guidelines; Department of Animal Welfare, Government of India.

Prediction of Biological activity
The biological activities were predicted by using Pass online software.
(http://www.pharmaexpert.ru/passonline/)

The concept of biological activity spectrum was introduced to describe the properties of biologically active substances. The PASS (Prediction of Activity Spectra for Substances) software product, which predicts more than 30000 pharmacological effects and biochemical mechanisms on the basis of the structural formula of a substance, may be effectively used to find new targets (mechanisms) for some ligands and conversely, to reveal new ligands for some biological targets. 51 Prediction of activity spectra for substances (PASS) is hosted by the V. N. Orechovich Institute of Biomedical Chemistry under the aegis of the Russian Foundation of Basic Research. The web based application predicts the biological activity spectrum of a compound based on its structure. It works on the principle that the biological activity of a compound equates to its structure. PASS prediction tools are constructed using 20000 principal compounds from MDDR (MDL (Molecular Design Laboratory) Drug Data Report) database (produced by Accelrys and Prous Science). The database contains over 180000 biologically relevant compounds and is constantly updated.

Evaluvation of antidepressant activity
Invivo study
Forced swimming test
The forced swimming test (FST) is used to test the behavioral despair in rodents. It can be seen as a way to measure "fighting spirit" of mice. In the first 2 min., the animal was allowed to adjust to the new conditions, then,

the immobility time that alternated with conditions of enhanced motor activity was measured. Immobility time was measured with a stopwatch for the next 4 minutes. Mice were removed from their cages and placed in individual glass cylinders (diameter 15 ml) containing water at 22-24°C at a depth of 14-16 cm so that they could not escape and could not touch the bottom. The animals were placed in the cylinders for observation in a 6-min test swim. Two swimming sessions were conducted: an initial 15-min pretest followed 24 h later by a 6-min test). The duration of immobility was measured for a 6-min period. The duration of immobility during the last 4 min. of the 6 min. test was measured by 2 trained experimenters. The mouse was considered as immobile when it stopped struggling and moved only to remain floating in the water, keeping its head above water. Shorter immobility time is an indicator of the stronger antidepressant effect of the test substance.

Tail suspension
The total duration of immobility time was also checked by the tail suspension test (TST) according to the method described as a means of evaluating potential antidepressants with slight modifications. Treatment was administered 30 min before the test, and then the mice were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. A test was conducted in 6 min and immobility time was calculated. Mice were considered immobile only if they hung passively and completely without any body movements.

Locomotor activity
The Photoactometer consisted of the square arena (30 * 30 * 25) with wire mesh bottom was used in this study. Six lights and six photocells were placed on the outer periphery of the bottom in such a way that the single mouse can block only one beam. Technically, its principle is that a photocell is activated when rays of light falling on the photocells are cut off by animals when crossing the beam of light. As the photocell activated count is recorded. The photocells are connected to an electronic automated counting device, which counts the number of "cut-offs." These cut-offs were counted for a period of 10 min, and the figure was considered as a measure of the locomotor activity of the animal.

Invitro study
Rt-pcr study
Isolation of total RNA (trizol method)
Total RNA was extracted from mice midbrain treated with drugs such as venlafaxine and venlafaxine with dextromethorphan. Total RNA was isolated using the total RNA isolation kit according to the manufacture instruction (Invitrogen – Product code10296010). Addition of Trizol solution causes the disruption of cells and the release of RNA. Chloroform extraction following centrifugation, exclusively in the aqueous phase whereas proteins are in the interphase and organic phase. On mixing with isopropanol, RNA gets precipitated as a

white pellet on the side and the bottom of the tube. Iml of trizol reagent was added of trizol reagent was added 100mg tissue and homogenised for 5 minutes. The contents were then transferred to a fresh sterile eppendorf tube. 200µl of chloroform was added and shaking was done vigorously for 15 seconds and incubated for 2-3minutes at room temperature, followed by centrifugation at 14000 rpm for 15 minutes at 4◦C. The aqueous layer was collected and 500 µl of100% isopropanol was added. It was incubated for 10 minutes at room temperature and then centrifuged at 14000 rpm for 15 minutes at 4◦C. Supernatant was discarded and pellet thus obtained was washed with 200 µl of 75% of ethanol (Merck). It was then centrifuged at 14000 rpm for 5 minutes at 4◦C in a cooling centrifuge (Remi CM12). The RNA pellet was dried and suspended in TE buffer.

Reverse transcriptase PCR analysis

Reverse transcription polymerase chain reaction (RT-PCR) is a variant of polymerase chain reaction laboratory commonly used in molecular biology to generate many copies of a DNA sequence, a process termed "amplification". In RT-PCR, however an RNA strand is first reverse transcribed into its DNA complement (Complementary DNA or cDNA) using the enzyme reverse transcriptase and the resulting cDNA is amplified using PCR or real time PCR. RT-PCR technique was performed using primer designed specifically for amplified gene.

i) cDNA Synthesis

The cDNA synthesis was performed using iScript™ cDNA Synthesis Kit (BIO-RAD). Product code 170-889). About 4µl of 5x iScript Reaction Mix, 1µl of iScript Reverse Transcriptase, 5 µl of RNA template (100fg to 1µg Total RNA) were added to an RNAse free tube Then making the total reaction volume up to 20 µl with the addition of sterile distilled water. The solution was mixed by pipetting gently up and down. The thermal cycler (Eppendorf Master Cycler) was programmed to undergo cDNA synthesis. The following cycling conditions were employed. 5 minutes at 25ºC, 30minutes at 42ºC and 5 minutes at 85ºC.

Primers used

Mice 5-HT serotin2A F 5'-CAACTCCA GAGATGCTAACACTTCG-3'
Mice 5-HT serotonin2A R 5'- GGGTTCTGGATGGCGACATAG-3'

GAPDH
Forward 5'AATGCATCCTGCACCACCAACTGC 3'
Reverse 5' GGAGGCCATGTAGGCCATGAGGTC 3'

ii) Amplification

The amplification was done using Thermoscientific amplification kit. The following components were added to a new PCR vial in a PCR work station. For each 50 µL reaction: 25 µL of PCR Master Mix (2X), 2 µLof

Forward primer (0.1-1.0 µM),2 µLof Reverse primer (0.1- 1.0 µM), 5 µLof Template DNA (10 pg - 1 µg). The components were made upto 50 µL with sterile distilled Water (nuclease-free). Initial denaturation at 95°C for 3 minutes, followed by denaturation at 95°C for 30s , annealing at Tm for 30 s and extension at 72°c for 1 minute which was repeated for 35 cycles and the final extension at 72°C for 5 minutes after the amplification.

iii) Agarose gel electrophoresis

The PCR product was separated by agarose gel electrophoresis after amplification. Agarose gel electrophoresis is a method for separating and visualizing DNA fragments. The fragments are separated by charge and size and move through agarose gel matrix, when subjected to an electric field. The electric field is generated by applying potential across an electrolyte solution (buffer).When boiled in an aqueous buffer, agar dissolve and upon cooling solidifies to a gel. 1.5% agarose gel was prepared in 1x TE buffer and melted in hot water bath at 90◦C.Then the melted agarose was cooled down to 45◦C. 6µl of 10 mg/ml of ethidium bromide was added and poured in to gel casting apparatus with the gel comb. After setting, the comb was removed from the gel. The electrophoresis buffer was poured in the gel tank and the platform with the gel was placed in it so as to immerse the gel. The gel was loaded with the samples and run at 50 V for 30 minutes. The stained gel was visualized using a gel documentation system (E gel imager, Invitrogen).

Statistical Analysis

The data were evaluated by one‑way analysis of variance followed by individual comparisons using Tukey's post‑hoc test. All results are shown as mean ± standard error of the mean. ED50 was calculated using Graph Pad Prism 7 Software developed by Graph Pad Software, Inc., USA.

RESULTS

Data Computed From PASS Software

Different biological activities of selected drugs were predicted using PASS software and the result obtained are summarized in Table 1 .From the PASS value obtained for dextromethorphan it is understood that it have antagonistic action on various nicotinic sub receptors like Nicotinic alpha4beta4 receptor, Nicotinic alpha6, beta3beta4alpha5 receptor, Nicotinic alpha2beta2 receptor with Pa values 0.748, 0.712, 0.618 respectively. If Pa> 0.7: chance to find the activity in experiments is very high, which shows that it is noncompetitive nicotinic receptor antagonist. From the PASS values obtained for venlafaxine it is clear that it have antidepressant activity with a Pa value of 0.618. Also it posess various other activities like Mood disorders treatment, 5 Hydroxytryptamine uptake inhibitor, Phobic disorders treatment, analgesic with Pa values 0.666, 0.660, 0.607, 0.525 respectively .If 0.5< Pa< 0.7: chance to find out the activity in experiment is less, but it had

the activity. The Pa value for the antidepressent activity of duloxetine was 0.634 other than this activity it also have Posttraumatic stress disorder treatment with Pa value 0.609, Mood disorders treatment with Pa value 0.634, 5 Hydroxytryptamine uptake inhibitor with Pa value 0.575, analgesic with Pa value 0.621. If $0.5< Pa< 0.7$: chance to find out the activity in experiment is less, but it had the activity. From the PASS value obtained for each drug it is clear that each drugs alone have the desired activity .So by combining these drugs it may produces the synergistic activity.

Forced Swim Test
The analysis of antidepressent activity of venlafaxine, duloxetine, dextromethorphan and combination of venlafexine and duloxetine with dextromethorphan were done by forced swim test. The mice were treated with different drugs such as venlafaxine, duloxetine, dextromethorphan, venlafexine with dextromethorphan, duloxetine with dextromethorphan. The control group animals show long duration of immobility. The venlafexine treated groups shows significant decrease in immobility compared with control group (P≤ 0.01). Simillarly the duloxetine treated group of shows significant reduction in immobility as compared to control group(P≤ 0.01). In dextromethorphan treated group there is significant reduction in immobility compared to control group but when comparing it with venlafexine and duloxetine the effect is less. Group of animal recieving the combination of dextromethorphan and venlafexine shows subadditive effect compared with group receiving venlafexine alone (P≤ 0.001). Simillarly the group recieving combination of dextromethorphan and duloxetine shows subaddditive effect compared to group recieving duloxetine alone (P≤0.001). The percentage decrease in immobility observed with venlafaxine, duloxetine, dextromethorphan, venlafaxine with dextromethorphan, duloxetine with dextromethorphan are in the order of 70.26%, 70.07%, 38.34%, 80.18%, 79.16% respectively compared to the control group. When compairing the percentage decrease in immobility time of each group it is clear that the groups recieving the combination of drugs have more activity than groups recieving single drug, which suggest that the nicotinic antagonist produces a synergistic action on the antidepressant drugs. The decrease in immobility time observed was summarized in Table 2 and Figure 1(a). The percentage decrease in immobility was shown in Table 3 and Figure 1(b).

Tail suspension test
The mice were treated with different drugs such as venlafaxine, duloxetine, dextromethorphan, venlafexine with dextromethorphan, duloxetine with dextromethorphan. The control group animals show long duration of immobility. The venlafexine treated groups shows significant decrease in immobility compared with control group (P≤0.01). Simillarly the duloxetine treated group of shows significant reduction in immobility as compared to control group (P≤0.01). In

dextromethorphan treated group there is significant reduction in immobility compared to control group but when comparing it with venlafexine and duloxetine the effect is less. Group of animal recieving the combination of dextromethorphan and venlafexine shows subadditive effect compared with group recieving venlafexine alone (P≤0.001). Simillarly the group recieving combination of dextromethorphan and duloxetine shows subadditive effect compared to group recieving duloxetine alone(P≤0.001).The percentage decrease in immobility observed with venlafaxine, duloxetine, dextromethorphan, venlafaxine with dextromethorphan, duloxetine with dextromethorphan are in the order of 70.283%, 66.618%,47.436%, 81.872%, 78.320% respectively compared to the control group. When compairing the percentage decrease in immobility time of each group it is clear that the groups recieving the combination of drugs have more activity than groups recieving single drug, which suggest that the nicotinic antagonist produces a synergestic action on the antidepressent drugs. The decrease in immobility time observed were summarized in Table4 and Figure 2(a). The percentage decrease in immobility were shown in Table 5 and Figure 2(b).

Locomotor activity
The effect of various drugs in locomotor activity in mice is checked by using actophotometer. The mice were treated with s such as venlafaxine (8mg/kg), duloxetine (30mg/kg), dextromethorphan (30mg/kg), venlafexine with dextromethorphan (4+15 mg/kg) and duloxetine with dextromethorphan (15+15 mg/kg). Venlafaxine and duloxetine does not show any significant effect on locomotor activity, so concluding the fact that the reduction in duration of immobility with these drugs was due to antidepressant effect and not because of false positive central nervous system stimulatory effect. While the groups treated with dextromethorphan show increase in locomotor activity (P≤0.01). which shows that dextromethorphan have significant effect in locomotor activity. Since dextromethorphan showed significant stimulant effects, a correlation analysis between locomotor activity and immobility time was carried out to determine whether stimulant effects could account for its apparent antidepressant-like actions. The Pearson's r correlation test revealed that there was no correlation between the dextromethorphan-induced increase in locomotor activity and decrease in immobility time. From the correlation it was concluded that the reduction in duration of immobility with these drugs was due to antidepressant effect and not because of false positive central nervous system stimulatory effect the change in locomotor activity observed were shown in Table 5 and Figure 3(a). The correlation analysis was shown in Figure 3(b).

Invitro study
Reverse transcriptase PCR analysis
Reverse transcription polymerase chain reaction (RT-PCR) is a variant of polymerase chain reaction

laboratory commonly used in molecular biology to generate many copies of a DNA sequence, a process termed "amplification". In RT-PCR, however an RNA strand is first reverse transcribed into its DNA complement (Complementary DNA or cDNA) using the enzyme reverse transcriptase and the resulting cDNA is amplified using PCR or real time PCR. RT-PCR technique was performed using primer designed specifically for amplified gene. Venlafaxine treating group of mice was selected for PCR study because when compairing the effect of duloxetine and venlafaxine, the group recieving venlafaxine show more activity in *invivo* tests. The brain tissue of mice treated with venlafaxine (8mg/kg) and combination of venlafaxine and dextromethorphan (4mg/kg+15mg/kg) was used for the study. The primer sequence used in study is Forward 5'-CAACTCCAGAGATGCTAACACTTCG-3' and Reverse 5'-GGGTTCTGGATGGCGACATAG-3' for Mice 5-HT serotin2A receptor. The primerse used for GAPDH are Forward 5'AATGCATCCTGCACCACC AACTGC 3' and Reverse 5' GGAGGCCATGTAG GCCATGAGGTC 3'. Where GAPDH was used as housekeeping gene and serves as a control From the result of the study it is understood that there will be a increase in seratonin receptor expression in mice treated with combination of venlafaxine and dextromethorphan when compared with mice treated with venlafaxine alone. The results of the study are shown in Figure 4 and 5.

Table 1: PASS value of drugs.

| Drug	Biologial activity	Pa value	Pi value
Dextromethorphan	Nicotinic alpha4beta4 receptor antagonist	0.748	0.013
	Nicotinic alpha6beta3beta4alpha5 receptor antagonist	0.712	0.031
	Nicotinic alpha2beta2 receptor antagonist	0.618	0.044
	Nicotinic receptor alpha7 subunit antagonist	0.213	0.005
	Acetylcholine neuromuscular blocking agent	0.621	0.016
	Antidepressant	0.517	0.019
Venlafaxine	Antidepressant	0.681	0.008
	Mood disorders treatment	0.666	0.009
	5 Hydroxytryptamine uptake inhibitor	0.660	0.004
	Phobic disorders treatment	0.607	0.120
	Analgesic	0.525	0.032
Duloxetine	Antidepressant	0.634	0.011
	Mood disorders treatment	0.634	0.010
	Posttraumatic stress disorder treatment	0.609	0.001
	5 Hydroxytryptamine uptake inhibitor	0.575	0.004
	Analgesic	0.621	0.017
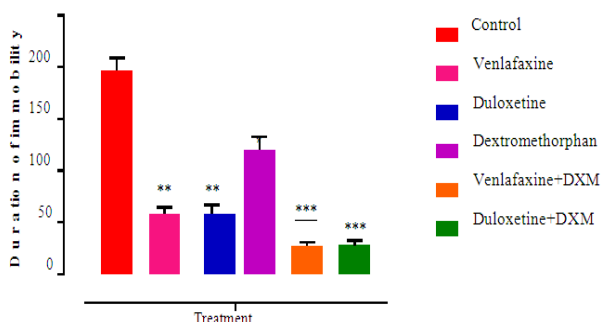
Figure 1 (a): Forced swim test in mice- Decrease in immobilityb.

Mice were treated with venlafaxine, duloxetine, dextromethorphan, venlafexine with dextromethorphan and duloxetine with dextromethorphan. All values are expressed as Mean ± SEM (n = 6). The statistical analysis was carried out by one way ANOVA followed by individual comparisons usingposthoc Tukey's multiple comparison tests where each group compared with that of control group. Where

****$P<0.0001$, ***$P<0.001$, **$P<0.01$, *$P<0.05$.

Table 2: Effects of Various Treatments on Forced Swimming Test in Mice.

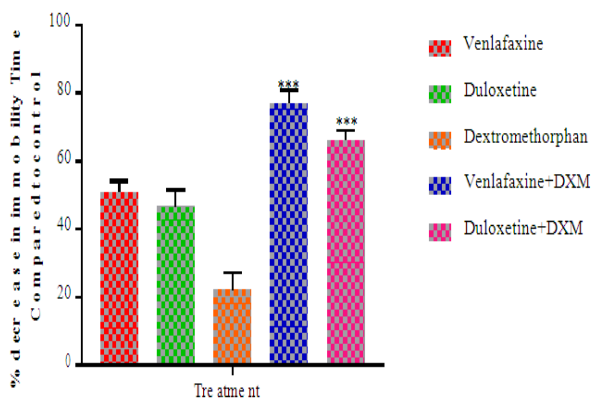| Group	Duration of immobility (Mean ±SEM)
Control	196.5±4.958
Venlafexine (8mg/kg)	58.33±2.459**
Duloxetine (30mg/kg)	58.333±3.373**
Dextromethorphan (30mg/kg)	92.833±3.081*
Venlafaxine+DXM (4+15 mg/kg)	27.333±1.453***
Duloxetine+DXM (4+15mg/kg)	28.000±1.897***

Figure 1(b): Forced swim test in mice-Percentage decrease in immobility.

All values are expressed as Mean±SEM (n=6). The statistical analysis was carried out by one way ANOVA followed by individual comparisons using posthoc Tukey's multiple comparison tests. Where each combination of group compared with that of single treatment group. Where ****$P<0.0001$, ***$P<0.001$, **$P<0.01$, *$P<0.05$.

Table 3: Percentage decrease in immobility.

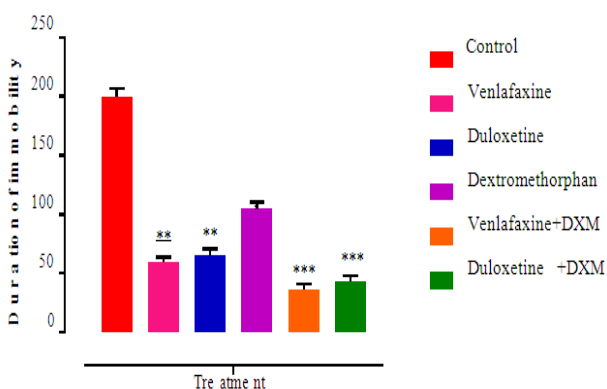| Group	Percentage decrease in immobility (%)
Control	-
Venlafexine (8mg/kg)	70.26
Duloxetine (30mg/kg)	70.07
Dextromethorphan (30mg/kg)	38.34
Venlafaxine+DXM (4+15 mg/kg)	80.18***
Duloxetine+DXM (15+15 mg/kg)	79.16***

Figure 2(a): Tail suspension test in mice-Decrease in immobility.

All values are expressed as Mean±SEM (n=6). The statistical analysis was carried out by one way ANOVA followed by individual comparisons using posthoc Tukey's multiple comparison tests where each group compared with that of control group.

Where****$P<0.0001$, ***$P<0.001$, **$P<0.01$, *$P<0.05$.

Table 4: Effects of Various Treatments on Tail suspension.

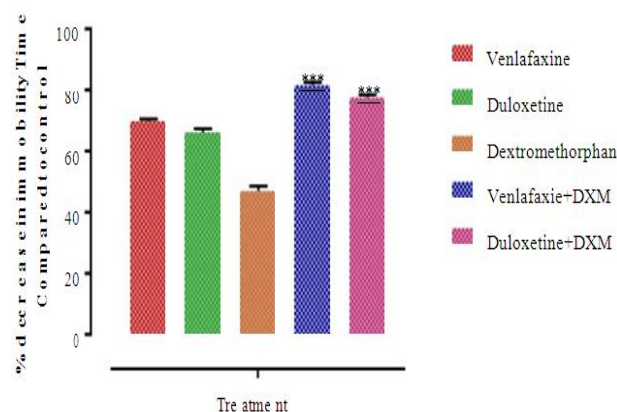| Group	Duration of immobility (Mean ±SEM)
Control	199.833±2.833
Venlafexine (8mg/kg)	60.167±1.447**
Duloxetine (30mg/kg)	66.000±1.983**
Dextromethorphan (30mg/kg)	104.833±2.315*
Venlafaxine+DXM (4+15mg/kg)	43.333±1.994***
Duloxetine+DXM (15+15mg/kg)	36.167±1.905***

Figure 2(b): Tail suspension test in mice-Percentage decrease in immobility.

All values are expressed as Mean±SEM (n=6).The statistical analysis was carried out by one way ANOVA followed by individual comparisons using posthoc Tukey's multiple comparison tests. Where each combination of group compared with that of single treatment group Where ****$P<0.0001$, ***$P<0.001$, **$P<0.01$, *$P<0.05$.

Table 5: Percentage decrease in immobility.

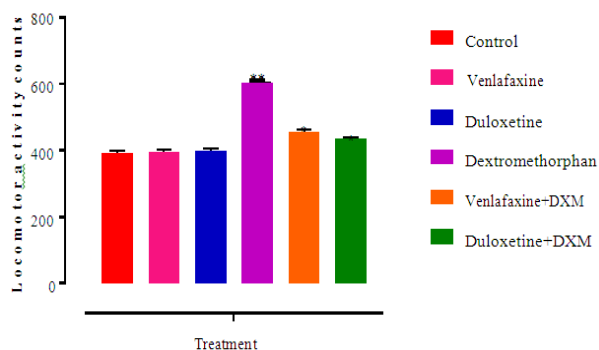| Group	Percentage decrease in immobility (%)
Control	-
Venlafexine (8mg/kg)	70.283
Duloxetine (30mg/kg)	66.618
Dextromethorphan (30mg/kg)	47.436
Venlafaxine+DXM (4+15mg/kg)	81.872***
Duloxetine+DXM (15+15mg/kg)	78.320***

Figure 3(a): Locomotor activity in mice.

All values are expressed as Mean ± SEM (n = 6). The statistical analysis was carried out by one way ANOVA followed by individual comparisons usingposthoc Tukey's multiple comparison tests Where each group compared with that of control group Where ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05.

Table 6: Locomotor activity count.

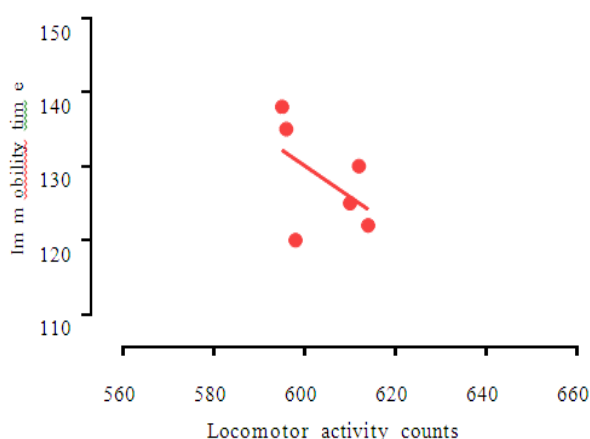| Group	Locomotor activity count (Mean ±SEM)
Control	391.167±3.049
Venlafexine (8mg/kg)	397.000±2.394
Duloxetine (30mg/kg)	399.333±2.871
Dextromethorphan (30mg/kg)	604.167±3.563**
Venlafaxine+DXM (4+15mg/kg)	457.333±2.092*
Duloxetine+DXM (15+15mg/kg)	434.833±1.515*

Figure 3(b): Correlation analysis of locomotor activity and immobility change of dextromethorphan.

The correlation study was done using the Pearson's r correlation test. The study reveled that that there was no correlation between the dextromethorphan-induced increase in locomotor activity and decrease in immobility time (r= -0.5082, n.s).
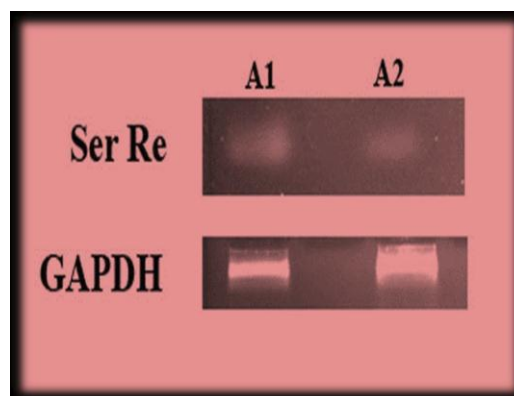

Figure 4: Expression of seratonin receptor in mice brain.

Mice were treated with venlafaxine (sample A2) and venlafaxine with dextromethorphan (sample A1). The housekeeping gene GAPDH was used as a control.

A1- Venlafaxine (4mg/kg) + Dextromethorphan (15mg/kg), A2- Venlafaxine (8mg/kg)

DISCUSSION

Mood disorders such as major depressive disorders (mdds) and bipolar disorder (manicdepressive illness) are common, severe, chronic and often life-threatening illness. More than 20% of the adult population suffers from these conditions at some time during their life. Suicide is estimated to be a cause of death in up to approximately 15% of individuals with mdds. In addition mdds represent a major risk factor for the development of cardiovascular disease and death after myocardial infarction. The World Health Organization (WHO) predicts that depression will become the second leading cause of premature death or disability worldwide by the year 2020.[11]

Antidepressantsare the most prescribed therapy for depression. The exact mechanism of action of antidepressants is unknown. The prevailing theory is that antidepressants increase the concentration of one or more brain chemicals (neurotransmitters). The neurotransmitters affected by antidepressants are norepinephrine, serotonin, and dopamine.[12] There are different types of antidepressants Monoamine oxidase inhibitors, Tricyclic antidepressants, Selective serotonin reuptake inhibitors, serotonin noradrenaline reuptake inhibitors. Both multi-target agents and polypharmacy ideally couple a therapeutically unexploited action to a clinically established mechanism in order to enhance efficacy, moderate side-effects, accelerate onset of action and treat a broader range of symptoms. Many patients with depression fail to derive sufficient benefit from available treatment options, with up to a third never reaching remission despite multiple trials of appropriate treatment. Novel antidepressant agents are needed, and drugs targeting nicotinic acetylcholine receptors (nAChRs) appear to hold promise in this regard. nAChRs

are involved in a variety of neurobiological systems implicated in the pathophysiology of depression.[13]

In the present study, FST and TST were used for behavioural studies as these are the most commonly used behavioural models for depression in mice. FST is a rodent behavioral test used for evaluation of antidepressant drugs, antidepressant efficacy of new compounds, and experimental manipulations that are aimed at rendering or preventing depressive-like states.[14] Mice are placed in an inescapable transparent tank that is filled with water and their escape related mobility behavior is measured. The control group animals show long duration of immobility. The venlafexine and duloxetine shows significant decrease in immobility compared with control. In case of dextromethorphan there will be significant reduction in immobility compared to control but when comparing it with venlafexine and duloxetine the effect is less.[15] The combination of venlafaxine and duloxetine with dextromethorphan shows more significant effect than the single drugs. which shows that the nicotinic antagonist dextromethorphan produces a synergestic action on the antidepressent drugs. The tail-suspension test is a mouse behavioral test useful in the screening of potential antidepressant drugs, and assessing of other manipulations that are expected to affect depression related behaviors. Mice are suspended by their tails with tape, in such a position that it cannot escape or hold on to nearby surfaces. The control group animals show long duration of immobility. The venlafexine and duloxetine shows significant decrease in immobility compared with control. In case of dextromethorphan there will be significant reduction in immobility compared to control but when comparing it with venlafexine and duloxetine the effect is less. The combination of venlafaxine and duloxetine with dextromethorphan shows more significant effect than the single drugs which shows that the nicotinic antagonist dextromethorphan produces a synergestic action on the antidepressant drugs.[16]

Locomotor activity was performed in all the groups for concluding the fact that the reduction in duration of immobility with these drugs was due to antidepressant effect and not because of false positive central nervous system stimulatory effect. Venlafaxine and duloxetine does not show any significant effect so concluding the fact that the reduction in duration of immobility with these drugs was due to antidepressant effect and not because of false positive central nervous system stimulatory effect while dextromethorphan show increase in locomotor activity. Which shows that it have significant effect in locomotor activity. Since dextromethorphan showed significant stimulant effects, a correlation analysis between locomotor activity and immobility time was carried out to determine whether stimulant effects could account for its apparent antidepressant-like actions. The Pearson's r correlation test revealed that there was no correlation between the dextromethorphan-induced increase in locomotor activity and decrease in immobility time.[17]

In patients with MDD, 5-HT1A receptor expression and activity are altered in raphe nuclei, hippocampus, and many cortical regions. The activation of 5-HT1A autoreceptors, through the binding of serotonin (or full or partial receptor agonists), initially produces an inhibition of serotonin release by the neuron. SNRIs inhibit serotonin reuptake through SERT, producing an increase in synaptic serotonin, which binds postsynaptic receptors found proximal to the synaptic cleft to increase serotonergic signaling. However, the increased serotonin also begins to bind more distal 5-HT1A autoreceptors.[18] Eventually, 5-HT1A autoreceptors desensitize or are down-regulated, removing the inhibitory signal and permitting synaptic serotonin levels to rise in the presence of sustained blockade of SERT by the SSRI. Ligands that bind to 5-HT1A autoreceptors and heteroreceptors, as antagonists (which block serotonin activity), have shown beneficial effects on depression symptoms, both clinically and in research situations. In the present study the brain tissue of mice treated with venlafaxine and combination of venlafaxine and dextromethorphan was studied. From the results obtained from RT-PCR the seratonin receptor expression expression in mice brain treated with venlafaxine with dextromethorphan is increased when compared with mice treated with venlafaxine alone.when seratonin receptor expression increases in the brain it will lead to reduced it produces beneficial effect on depression. So it is clear that the concurrent usage of a nicotinic receptor antagonist increase the effect of antidepressent drugs.[19]

Dextromethorphan blocks the nicotinic receptor function in a noncompatitive manner suggesting that the drug block the receptor channel. These datas indicating that this drug can be used as a antagonist of nicotinic receptor. Two reports indicate that dextromethorphan is a functional antagonist of nicotinic receptor. It reduces the release of acetylcholineby blocking the nicotinicreceptors. The cholinergic hypothesis of depression proposes that hyperactivity of the cholinergic system over that of the adrenergic system leads to depression.[20]

Several lines of evidence from rodent and human studies support this hypothesis. In humans, physostigmine, which potentiates cholinergic transmission by inhibiting acetylcholinesterase (AChE),[21] the enzyme that breaks down ACh, produces depressive-like symptoms in individuals with and without a history of depression. Administration of the nonselective nicotinic antagonist demonstrated putative antidepressant-like effects, especially in treatment-resistant. The cholinergic hypothesis of depression postulates a hyperactivity of the cholinergic system over that of the adrenergic system in the brain.[22] Choline (the rate-limiting precursor to endogeneous ACh) crosses the blood−brain barrier to enter the brain and is actively transported into the

cholinergic presynaptic terminals by an active uptake mechanism. The neurotransmitter ACh is synthesized from choline and acetyl coenzyme A, catalyzed by the enzyme choline acetyl transferase. ACh is sequestered into secretory vesicles by vesicular Ach transporters. Once released from the presynaptic terminals, ACh can interact with a variety of presynaptic and postsynaptic receptors. Two classes of the cholinergic ACh receptors are muscarinic (G proteincoupled) and nicotinic (ionotropic). Once activated, nAChRs form transient open cationic channels that allow the ions Na+, K+, and Ca2+ to flow across the plasma membrane and induce cellular responses. Prolonged exposure to ACh or nicotinic agonist causes a gradual decrease in the rate of this ionic response, leading to a high affinity, longer-lasting functionally inactive state, referred to as desensitization.[23] ACh has its signal terminated primarily by the enzyme AChE, unlike many other monoaminergic neurotransmitters where reuptake mechanisms predominate. A recent human imaging study has suggested that acetylcholine (ACh) levels are elevated in patients who are actively depressed, as measured by occupancy of nicotinic receptors throughout the brain, and remain high in patients who have a history of depression.[24] In addition, despite the recent failure of a large clinical trial, other clinical and preclinical studies have shown that blockers of cholinergic (both muscarinic and nicotinic) receptors can induce antidepressant-like responses.[25]

CONCLUSION

Behavioral studies indicated that antidepressant venlafaxine and duloxetine with dextromethorphan resulted in significant reduction in immobility time compared to the vehicle control in FST and TST, respectively. Locomotor activity studies demonstrated no significant increase in general locomotion after co-administration of the compounds which shows that the reduction in duration of immobility with these drugs was due to antidepressant effect and not because of false positive central nervous system stimulatory effect. The RT-PCR study reveals that the coadministration of dextromethorphan with antidepressents will increase the receptor expression and thereby increase the effect. The study results suggest that co-administration of dextromethorphan which is a nicotinic receptor antagonist with venlafaxine and duloxetine increases the antidepressive effects with the reduction of dose.

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