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EVALUATION OF THE EFFICACY OF COMBINATION THERAPY OF AGOMELATINE, DULOXETINE AND SERTRALINE IN THE MANAGEMENT OF STRESS INDUCED DEPRESSION

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Keywords:

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ABSTRACT: There is unmet need in the treatment of stress induced depression on suggesting requirement of new therapeutic agent. A combination therapy of antidepressants sertraline (5 mg/kg), duloxetine (10 mg/kg) and agomelatine (8 mg/kg) are used in different combinations of doses by intra-peritoneal route. Swiss albino mice were used as an animal model for stress-induced depression; mice were divided into eight groups. Depression was induced through forced swim test, tail suspension test and sucrose preference test (chronic mild stress model). Efficacy of combination therapy was analyzed in specific regions of mice brain associated with depression (hippocampus, cerebral cortices and the whole brain) through brain monoamine estimation by HPLC (High-performance liquid chromatography) with fluorescence detector. Three drug combination [Agomelatine (2.67 mg/kg) plus duloxetine (3.33 mg/kg) plus sertraline (1.67 mg/kg)] showed antidepressant activity in *in-vivo* animal model testing parameters (decrease in immobility period, increase in swimming count and climbing count and increase sucrose consumption) than other treated group. In the same group, improvement in brain monoamine profile was observed illustrating antidepressant activity (increasing dopamine, serotonin and norepinephrine level in hippocampi, cerebral cortex and whole brain). These findings could be further probed with the aim of understanding the interaction between agomelatine plus duloxetine plus sertraline as a future endeavour.

INTRODUCTION: Depression is a common mental disorder that affects person's behaviour, thoughts, feelings, and physical well - being [25]. A diagnosis of depression might specify one of two primary types: Unipolar depression or bipolar depression.

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1. Unipolar depression further differentiated as, Major depression, Dysthymic disorder, or Dysthymia and Minor depression.

2. Bipolar disorder, psychotic depression, postpartum depression, seasonal affective disorder (SAD).

Sertraline is serotonin selective reuptake inhibitors (SSRIs). Sertraline is primarily a serotonin reuptake inhibitor (SRI) with a binding affinity towards the serotonin transporter. It inhibits the CNS neuronal reuptake; it also acts as a dopamine reuptake inhibitor.

Duloxetine is a potent serotonin and norepinephrine reuptake inhibitor [24]. The dual action makes it an interesting drug in the treatment of depression [9, 19, 20]. Agomelatine, a naphthalene analog of melatonin, is a newly developed selective agonist of the human cloned melatonergic MT1 and MT2 receptors [15]. Agomelatine also shows serotonin 5-HT2C receptor antagonist activity [15]. Furthermore, *in vivo* data indicate that agomelatine enhances the levels of dopamine and noradrenaline in frontal cortex, but not in nucleus accumbens or striatum, probably secondary to blockade of the inhibitory input of 5 HT2C receptors to cortical dopaminergic and adrenergic pathways [26]. Agomelatine has a significant impact on the sleep of patients with MDD, with a positive effect on the subjective reports of sleep quality and a shortening of sleep latency. These changes in sleep patterns seem to occur as early as the first week of treatment and precede the changes in HAM-D score [6].

Objective: To assess the augmentation approach of a three drug combination melatonergic anti-depressant (agomelatine), selective serotonin reuptake inhibitor (sertraline), serotonin-norepine-phrine reuptake inhibitor (duloxetine) in the treatment of stress induced depression using various antidepressant model.

MATERIALS AND METHOD:

Animals: Male Swiss Albino mice weighing 22 - 27 gm were procured from Bharat Serums and Vaccines Pvt. Ltd., Thane, Bombay Veterinary College. They were housed in groups of 6 in polycarbonate cages at SVKM's animal facility (12h: 12h light/dark cycle, room temperature 20 - 22 °C and humidity 70 ± 5%). They had free access to standard food and water. Animals were allowed to adapt themselves to the new environment for one week prior to the start of the experimental works. Experiments were approved by an Institutional animal ethical committee for the use of Animal Subjects (Approval Number – CPCSEA/IAEC/ BNCP/P-03/2015) and (CPCSEA/IAEC/P-13/20 16). Experiment were performed between 10.00 a.m. to 6.00 p.m. Animal model like tail suspension test and forced swim test were conducted on same set of animals, whereas sucrose preference test was conducted on another set of animals. In each animal model, animals were randomly distributed into 8 groups (n = 6 / group).

Drug Solutions and Treatment: Drug was administered through intra-peritoneal route. Sertraline was dissolved in distilled water, duloxetine was dissolved in normal saline (0.9% w/v NaCl solution) and Agomelatine was homogeneously suspended in 1% solution of hydroxyl ethyl cellulose. Drug solutions were prepared just before each injection session. Each animal model has 8 groups with 6 animals per group. Control group (Group I) received normal saline (5 ml/kg). Treatment of sertraline (5 mg/kg; Cipla Pvt. Ltd.,) Group II, duloxetine (10 mg/kg; TCI chemicals) Group III, and agomelatine (8 mg/kg; Watson Pharmaceuticals Pvt. Ltd.,) Group IV, and combination of agomelatine (4 mg/kg) + duloxetine (5 mg/kg) Group V, sertraline (2.5 mg/kg) + duloxetine (5 mg/kg) Group VI, sertraline (2.5 mg/kg) + agomelatine (4 mg/kg) Group VII, and Three drug combination, Group VIII; *i.e.* agomelatine (2.67 mg/kg) + duloxetine (3.33 mg/kg) + sertraline (1.67 mg/kg).

Antidepressant Models:

Forced Swim Test (FST): The forced swim test (FST) paradigm has been described previously [18]. It was suggested that mice forced to swim in a restricted space from which they cannot escape are induced to a characteristic behaviour of immobility. This behaviour reflects a state of despair which can be reduced by several agents which are therapeutically effective in human depression. The test was performed 30 min after administration of drug. In brief, mice underwent ''pretest session" one day prior to main tests. They were individually forced to swim for 15 min in a Plexiglas cylinder (10 cm diameter × 25 cm height) with water (22 - 24 °C) at a depth of 18 cm. On next day mice was allowed to swim for 6 min and the entire act was video-taped. Last 5 min session from total 6 min recorded video was used for evaluation. Immobility refers to the cessation of struggling and remaining motionless in the water, making small movements needed to keep the animal's head above the water [18]. After the tests, all animals were dried before returning to the home cage. The Immobility period, Swimming, climbing count was evaluated.

Tail Suspension Test (TST): The immobility displayed by rodents when subjected to an unavoidable and inescapable stress has been hypothesized to reflect behavioral despair which in

turn may reflect depressive disorders in humans. Clinically effective antidepressants reduce the immobility that mice display after active and unsuccessful attempts to escape when suspended by the tail. The test was performed for 30 min after administration of drug. Mice were suspended 58 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail [22]. The duration of immobility is recorded for a period of 5 min. Mice is considered immobile when they hang passively and completely motionless. The immobility period was evaluated.

Sucrose Preference Test: Intake and preference for sucrose solutions are the exact measures of anhedonia commonly used and accepted in the CMS literature [8]. All mice were kept in individual cages and were subjected to chronic mild stress protocol. They were trained to consume 4% w/v sucrose solution before the start of the CMS protocol. Mice were exposed to stress for 2 weeks and the weekly stress regimen consisted of Food deprivation, water deprivation, 45° cage tilt, overnight illumination, wet bedding. All mice were dosed daily for 14 days and on 7th and 14th day sucrose intake was calculated. The weekly CMS protocol was followed as described with slight modification is given in following table:

TABLE 1: CHRONIC MILD STRESS PROTOCOL

Day	Time	Task
Day 1	10.30 am	Cleaning of cages followed by no stress
	4.30 pm	Food and water deprivation
Day 2	10.30 am	Food water provided followed by no stress
	4.30 pm	Tilting of cage to 45°
Day 3	10.30 am	Cage brought to normal position followed by wet bedding
	4.30 pm	Wet bedding cage subjected to continuous light illumination
Day 4	10.30 am	Cage cleaning followed by stress
	4.30 pm	Food and water deprivation
Day 5	10.30 am	Food water provided followed by tilting of cage to 45°
	4.30 pm	Cage brought to normal position followed by continuous illumination
Day 6	10.30 am	Food and water deprivation
	4.30 pm	Food water provided followed by no stress
Day 7	12.00 am	Sucrose intake calculated

Brain Monoamine Estimation by HPLC with Fluorescence Detector (HPLC–FD) Method: Heads were dropped in ice cold perchloric acid (0.1M) immediately after euthanasia. After weighing brain, separation of cerebral cortex, hippocampus, and remaining brain parts were separated, weighed, and homogenized in 2 ml of ice cold 0.1 M perchloric acid. Analysis of monoamine levels in cerebral cortex, hippocampus, and whole brain (whole brain = cerebral cortex + hippocampus + remaining brain tissue) was performed using method described by [10] (HPLC – Shimadzu, LC-2010C HT, auto sampler with FD-RF -20A- prominence, Shimadzu). The method was optimized in house. Homogenized mixture was centrifuged at 16356 × g (Eppendorf 5810 R, Rotor F-45-30-11) for 30 min (4 °C) and the obtained supernatant was filtered through 0.45 µm membrane. Filtered supernatant was stored at -80°C until the time of analysis. After sample injection, the chromatographic separation was achieved on reversed-phase analytical column (Waters, C18, 5 µm, 25 mm × 0.46 mm) at room temperature. LC solution software was used to process acquired data.

The mobile phase was prepared using 0.36 gm of potassium dihydrogen orthophosphate, 0.5 ml of phosphoric acid dissolved in 1 ltr of Millipore water, sonicated and filtered through a 0.45 µm membrane. Flow rate of mobile phase was kept at 0.8 ml/min. Dopamine, serotonin and nor-epinephrine was detected at an excitation wavelength of 280 nm and an emission wavelength of 315 nm. Monoamine peaks were identified by comparing the retention time of sample and standard. The concentration of each monoamine in the sample was analysed according to their area under curve and using respective straight line equation. The linearity for Dopamine, serotonin and norepinephrine was in the range 0.99 - 0.997. Results were expressed as µg/g of wet weight of tissue [4, 13].

Statistical Analysis: The statistical evaluation was performed using the Graphpad Instat for 32 bit Windows version. Groups were compared to assess the statistical significance using one way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) post-hoc test. Data was represented as Mean ± SEM values and n = 6 per group.

RESULTS:

Forced Swim Test:

Immobility Period: All drug treated groups showed statistically significant decrease in immobility period as compared to control group

Fig. 1. Combination treated group Agomelatine (4 mg/kg) + Duloxetine (5 mg/kg) showed significant decrease in immobility period, as compared to 'Agomelatine alone' (8 mg/kg), 'Duloxetine alone' (10 mg/kg) treated groups **Fig. 1**. Combination of Sertraline (2.5 mg/kg) + Duloxetine (5 mg/kg) showed significant decrease in immobility period, as compared to 'Sertraline alone' (5 mg/kg) and 'Duloxetine alone' (10 mg/kg) treated group **Fig. 1**. The immobility period was significantly decreased in Sertraline (2.5 mg/kg) + agomelatine (4 mg/kg) treated group when compared against 'Sertraline alone' (5 mg/kg) and 'agomelatine alone' (8 mg/kg) treated groups **Fig. 1**. However, three drug in combination *i.e.*, Agomelatine + Duloxetine + Sertraline (2.67 mg/kg + 3.33 mg/kg + 1.67 mg/kg) showed significant decrease in immobility period as compared to control and other combination treated group.

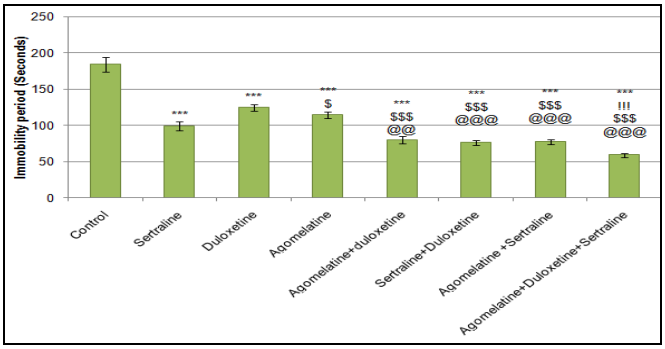

FIG. 1: IMMOBILITY PERIOD IN FORCED SWIM TEST

Significant difference is denoted by ***p<0.001 as compared against control group. !!!p<0.001 as compared to sertraline, $$$p<0.001, $p<0.05 as compared against duloxetine. @@@p<0.001, @@p<0.01 as compared against agomelatine group.

TABLE 2: IMMOBILITY PERIOD IN FORCED SWIM TEST

Group no.	Treatment	Immobility Period (Seconds)
1	Control	184.33 ± 9.939
2	Sertraline (5 mg/kg)	99.33 ± 6.386 ***
3	Duloxetine (10 mg/kg)	124.5 ± 4.890 ***
4	Agomelatine (8 mg/kg)	114.5 ± 4.610 *** $
5	Agomelatine + Duloxetine (4 mg/kg + 5 mg/kg)	79.83 ± 4.983 *** $$$@@
6	Sertraline + Duloxetine (2.5 mg/kg + 5 mg/kg)	76.167 ± 3.321 *** $$$@@@
7	Sertraline + Agomelatine (2.5 mg/kg + 4 mg/kg)	77.66 ± 3.293 *** $$$@@@
8	Agomelatine + Duloxetine + Sertraline (2.67 mg/kg + 3.33 mg/kg + 1.67 mg/kg)	59.167 ± 2.713 ***!!! $$$@@@

Swimming and Climbing Count: All drug treated groups showed statistically significant increase in swimming and climbing counts compared to control group **Fig. 2** and **3**. Both swimming and climbing counts was significantly increased in agomelatine (4 mg/kg) + duloxetine (5 mg/kg) treated group when compared against 'agomelatine alone' (8 mg/kg) and 'duloxetine alone' (10 mg/kg) treated group **Fig. 2** and **3**. Sertraline (2.5 mg/kg) + duloxetine (5 mg/kg) treated group when compared against 'sertraline alone' (5 mg/kg) and 'duloxetine alone' (10 mg/kg) treated groups **Fig. 2** and **3**.

Combination treated group sertraline (2.5 mg/kg) + agomelatine (4 mg/kg) showed significant increase in swimming and climbing counts, when compared against 'sertraline alone' (5 mg/kg) and 'agomelatine alone' (8 mg/kg) treated group **Fig. 2** and **3**. However, Three drug in combination *i.e.* agomelatine (2.67 mg/kg) + duloxetine (3.33 mg/kg) + sertraline (1.67 mg/kg) showed significant increase in swimming and climbing count as compared to control group and other combination treated group **Fig. 2** and **3**.

TABLE 3: SWIMMING AND CLIMBING COUNTS IN FORCED SWIM TEST

Group no.	Treatment	Swimming (No. of counts)	Climbing (No. of counts)
1	Control	3.167 ± 0.4773	3.33 ± 0.4216
2	Sertraline (5 mg/kg)	16.333 ± 2.333 ***	15.5 ± 1.057 ***
3	Duloxetine (10 mg/kg)	9.167 ± 0.8724 #	11.667 ± 1.764 **
4	Agomelatine (8 mg/kg)	10 ± 0.5774 *	13.833 ± 1.108 ***
5	Agomelatine + Duloxetine (4 mg/kg+ 5 mg/kg)	14.5 ± 1.586 ***	16.33 ± 1.944 ***
6	Sertraline + Duloxetine (2.5 mg/kg+ 5 mg/kg)	18.33 ± 1.726 ***$$@@	18.667 ± 1.333 ***$
7	Sertraline + Agomelatine (2.5 mg/kg + 4 mg/kg)	18 ± 1.065 *** $$$@	18.33 ± 2.040 *** $
8	Agomelatine + Duloxetine + Sertraline (2.67 mg/kg + 3.33 mg/kg + 1.67 mg/kg)	22.667 ± 2.140 ***$$$@@@!!	22.167 ± 1.352 ***#$$$@@

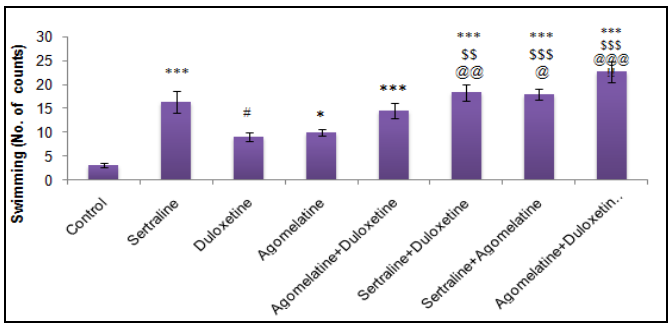

FIG. 2: SWIMMING COUNTS IN FORCED SWIM TEST

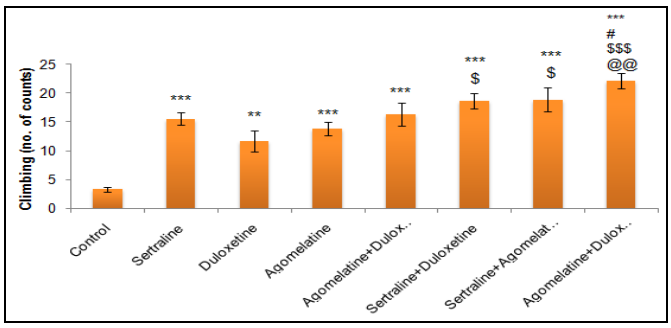

FIG. 3: CLIMBING COUNTS IN FORCED SWIM TEST

Significant difference is denoted by***P<0.001,*P<0.05 as compared against control group. #P<0.05 as compared against sertraline treated group. $$$P<0.001, $$P<0.01 as compared against duloxetine treated group. @@@P<0.001, @@P<0.01, @P<0.05 as compared against agomelatine treated group. !!P<0.01 as compared against agomelatine + duloxetine treated group.

Tail Suspension Test: The immobility period was significantly decreased in drug treated groups when compared against control group **Fig. 4**. Combination treated group agomelatine (4 mg/kg) + duloxetine (5 mg/kg) showed significant decrease in immobility period, as compared to 'agomelatine alone' (8mg/kg) and 'duloxetine alone' (10 mg/kg) treated groups **Fig. 4**. Combination of sertraline (2.5 mg/kg) + duloxetine (5 mg/kg) showed significant decrease in immobility period, as compared to 'sertraline alone' (5 mg/kg) and 'duloxetine alone' (10 mg/kg) treated group **Fig. 4**. Combination of sertraline (2.5 mg/kg) + agomelatine (4 mg/kg) showed decrease in immobility period, as compared to 'sertraline alone' (5 mg/kg) and 'agomelatine alone' (8 mg/kg) **Fig. 4**. However, three drug in combination *i.e.*, agomelatine (2.67 mg/kg) + duloxetine (3.33 mg/kg) + sertraline (1.67 mg/kg) showed significant decrease in immobility period, as compared against control group and other combination treated group **Fig. 4**.

TABLE 4: TAIL SUSPENSION TEST

S. no.	Treatment	Immobility Period (Seconds)
1	Control	191.83 ± 11.569
2	Sertraline (5 mg/kg)	99.167 ± 8.89***
3	Duloxetine (10 mg/kg)	133.5 ± 7.578***#
4	Agomelatine (8 mg/kg)	110.167 ± 5.452***
5	Agomelatine + Duloxetine (4 mg/kg + 5 mg/kg)	87.67 ± 2.996***$$$
6	Sertraline + Duloxetine (2.5 mg/kg + 5 mg/kg)	74.33 ± 4.41***$$$@@
7	Sertraline + Agomelatine (2.5 mg/kg + 4 mg/kg)	74.5 ± 4.137***$$$@
8	Agomelatine + Duloxetine + Sertraline (2.67 mg/kg + 3.33 mg/kg + 1.67 mg/kg)	50.5 ± 2.918***###$$$@@@!!!

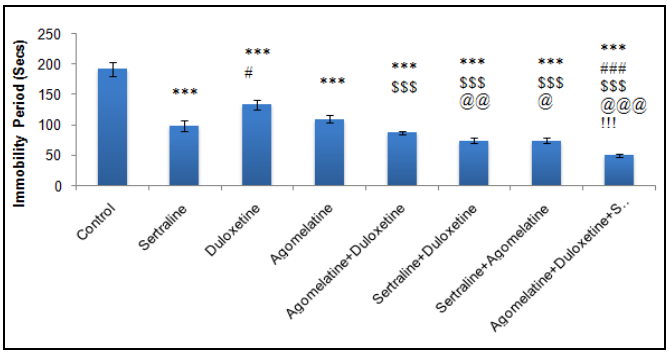

FIG. 4: TAIL SUSPENSION TEST

Significant difference is denoted by ***P<0.001 as compared against control group. ###P<0.001, #P<0.05 as compared against sertraline treated group. $$$P<0.001 as compared against duloxetine treated group. @@@P<0.001, @@P<0.01, @P<0.05 as compared against agomelatine treated group. !!!P<0.001 as compared against agomelatine + duloxetine treated group.

Sucrose Preference Test: On 14th day all drug treated groups showed statistically significant increase in amount of sucrose consumed as compared to control group **Fig. 5**. Combination of agomelatine (4 mg/kg) + duloxetine (5 mg/kg) showed significant increase in amount of sucrose consumed as compared to 'agomelatine alone' (8 mg/kg) and 'duloxetine alone' (10 mg/kg) treated groups **Fig. 5**. The amount of sucrose consumed was significantly increase in sertraline (2.5 mg/kg) + duloxetine (5 mg/kg) treated groups when compared against 'sertraline alone' (5 mg/kg) and 'duloxetine alone' (10 mg/kg) treated group **Fig. 5**. Combination treated group sertraline (2.5 mg/kg) + agomelatine (4 mg/kg) showed significant increase in amount of sucrose consumed as compared to

'sertraline alone' (5 mg/kg) and 'agomelatine alone' (8 mg/kg) treated groups **Fig. 5**. However, three drug in combination treated group *i.e.*, agomelatine (2.67 mg/kg) + duloxetine (3.33 mg/kg) + sertraline (1.67 mg/kg) showed significantly increase in amount of sucrose consumed as compared to control group and other combination treated group **Fig. 5**. Statistically significant difference was not obtained on the amount of sucrose consumed on 7th day **Fig. 5**.

TABLE 5: SUCROSE PREFERENCE TEST

Group no.	Treatment	Amount of Sucrose Consumed (gm) on 7th Day	Amount of Sucrose Consumed (gm) on 14th Day
1	Control	1.53 ± 0.108	1.6 ± 0.088
2	Sertraline (5 mg/kg)	2.14 ± 0.189	2.35 ± 0.116 *
3	Duloxetine (10 mg/kg)	2.01 ± 0.161	2.09 ± 0.167
4	Agomelatine (8 mg/kg)	2.39 ± 0.092	2.47 ± 0.111 ***
5	Agomelatine + Duloxetine (4 mg/kg + 5 mg/kg)	2.58 ± 0.125	2.71 ± 0.135 ***
6	Sertraline + Duloxetine (2.5 mg/kg +5 mg/kg)	2.62 ± 0.158	2.94 ± 0.19 *** @@
7	Sertraline + Agomelatine (2.5 mg/kg + 4 mg/kg)	2.66 ± 0.162	3.12 ± 0.2 *** $@@@
8	Agomelatine + Duloxetine + Sertraline (2.67 mg/kg + 3.33 mg/kg + 1.67 mg/kg)	4.50 ± 0.228	5.04 ± 0.187 *** $$$@@@!!! ### ++ +^^^

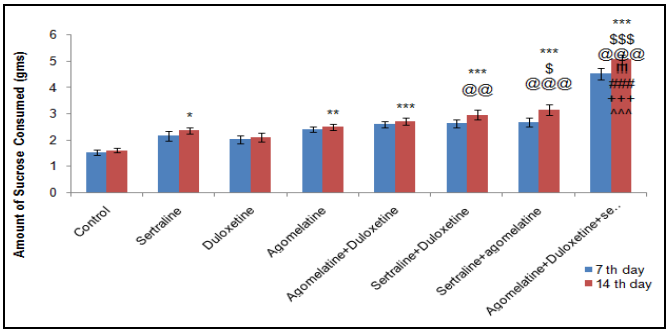

FIG. 5: SUCROSE PREFERENCE TEST

Significant difference is denoted by *** P<0.001, **P<0.01, *P<0.05 as compared against control group. $$$P<0.001, $P<0.05 as compared against sertraline treated group. @@@P<0.001, @@P<0.01 as compared against duloxetine treated group. !!!P<0.001 as compared against agomelatine treated group. ###P<0.001 as compared against agomelatine + duloxetine treated group. +++P<0.001 as compared against sertraline + duloxetine treated group. ^^^P<0.001 as compared against sertraline + agomelatine treated group.

Estimation of Dopamine, Norepinephrine and Serotonin by HPLC with Fluorescence Detector (HPLC-FD) Method:

Estimation of Dopamine Level:

Hippocampi: The drug treatments such as 'sertraline alone' (5 mg/kg), 'duloxetine alone' (10 mg/kg), 'agomelatine alone' (8 mg/kg), agomelatine (4 mg/kg) + duloxetine (5 mg/kg), sertraline (2.5 mg/kg) + duloxetine (5 mg/kg), sertraline (2.5 mg/kg) + agomelatine (4 mg/kg), agomelatine (2.67 mg/kg) + duloxetine (3.33 mg/kg) + sertraline (1.67 mg/kg) have shown significant increase in dopamine level when compared against control group **Fig. 6**. Agomelatine (4 mg/kg) + duloxetine (5 mg/kg) showed significant increase in dopamine levels, as compared to 'agomelatine alone' (8 mg/kg) and 'duloxetine alone' (10 mg/kg) treated group **Fig. 6**. Sertraline (2.5 mg/kg) + duloxetine (5 mg/kg) showed significant increase in dopamine level when compared against 'sertraline alone' (5 mg/kg) and 'duloxetine alone' (10 mg/kg) treated groups **Fig. 6**. Sertraline (2.5 mg/kg) + agomelatine (4 mg/kg) showed significant increase in dopamine level when compared against 'sertraline alone' (5 mg/kg) and 'agomelatine alone' (8 mg/kg) treated group **Fig. 6**. Three drug in combination i.e. agomelatine (2.67 mg/kg) + duloxetine (3.33 mg/kg) + sertraline (1.67 mg/kg) showed significant increase in dopamine level when compared against control group and other combination treated group **Fig. 6**.

Cerebral Cortices: Dopamine levels was significantly increased in agomelatine (4 mg/kg) + duloxetine (5 mg/kg) treated group as compared to 'agomelatine alone' (8 mg/kg) and 'duloxetine alone' (10 mg/kg) treated group **Fig. 6**.

Combination of sertraline (2.5 mg/kg) + duloxetine (5 mg/kg) treated group showed significant increase in dopamine levels, as compared to 'sertraline alone' (5 mg/kg) and 'duloxetine alone' (10 mg/kg) treated group **Fig. 6**. Combination of sertraline (2.5 mg/kg) + agomelatine (4 mg/kg) treated group showed significant increase in dopamine levels, as compared to 'sertraline alone' (5 mg/kg) and 'agomelatine alone' (8 mg/kg) treated group **Fig. 6**. Dopamine level was significant increase in three drug combination *i.e.*,

agomelatine (2.67 mg/kg) + duloxetine (3.33 mg/kg) + sertraline (1.67 mg/kg) treated group as compared to control group and other combination treated groups **Fig. 6**.

Whole Brain: The dopamine level was significantly increased in 'agomelatine (4 mg/kg) + duloxetine (5 mg/kg) treated group as compared to 'agomelatine alone' (8 mg/kg) and 'duloxetine alone' (10 mg/kg) treated group **Fig. 6**. Combination of Sertraline (2.5 mg/kg) + duloxetine (5 mg/kg) treated group showed significant increase in dopamine levels, as compared to 'sertraline alone' (5 mg/kg) and 'duloxetine alone' (10 mg/kg) treated group **Fig. 6**. Combination treated group sertraline (2.5 mg/kg) + agomelatine (4 mg/kg) showed significant increase in dopamine levels, as compared to 'sertraline alone' (5 mg/kg) and 'agomelatine alone' (8 mg/kg) treated group **Fig. 6**. The dopamine level was significantly increased in three drug combination *i.e.*, agomelatine (2.67 mg/kg) + duloxetine (3.33 mg/kg) + sertraline (1.67 mg/kg) treated group as compared against control group and other combination treated group **Fig. 6**.

TABLE 6: ESTIMATION OF DOPAMINE LEVELS IN HIPPOCAMPI, CEREBRAL CORTICES AND WHOLE BRAIN BY HPLC-FD

Group no.	Treatment	Dopamine levels µg/gm of wet weight of tissue		
		Hippocampi	Cerebral Cortices	Whole Brain
1	Control	8.443 ± 1.385	16.852 ± 1.117	29.611 ± 2.576
2	Sertraline	19.282 ± 0.3505	30.916 ± 6.588	39.353 ± 6.644
3	Duloxetine	15.478 ± 1.721	27.845 ± 4.447	48.411 ± 7.715
4	Agomelatine	25.068 ± 2.871**	38.052 ± 5.387	60.638 ± 11.194
5	Agomelatine + Duloxetine	23.192 ± 2.052*	39.735 ± 6.055	82.897 ± 5.194
6	Sertraline + Duloxetine	29.437 ± 4.713***$	44.162 ± 7.545*	99.52 ± 14.5**#
7	Sertraline + Agomelatine	30.796 ± 4.841***$	45.383 ± 6.117*	110.686 ± 13.274**##$
8	Agomelatine + Duloxetine + Sertraline	40.8336 ± 3.458***###$$$@&&	73.663 ± 7.442 ***###$$$@@&&+^	152.163 ± 26.27 ***###$$$@@@&

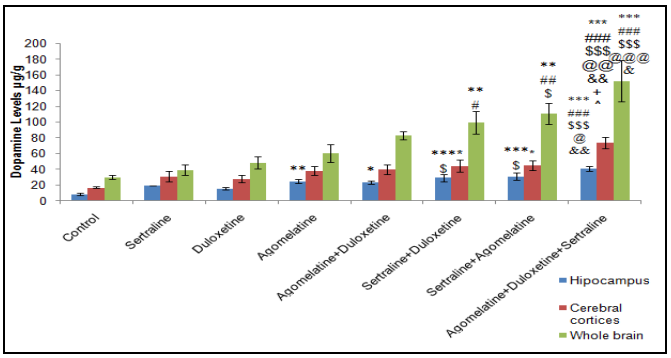

FIG. 6: ESTIMATION OF DOPAMINE LEVEL IN HIPPOCAMPI, CEREBRAL CORTICES AND WHOLE BRAIN BY HPLC-FD

Significant difference is denoted by ***P<0.001, **P<0.01, *P<0.05 as compared against control group. ###P<0.001, ##P<0.01, #P<0.05 as compared against sertraline treated group. $$$P<0.001, $P<0.05 as compared against duloxetine treated group. @@@P<0.001, @@P<0.01, @P<0.05 as compared against agomelatine treated group. &&P<0.01, &P<0.05 as compared against agomelatine + duloxetine treated group. +P<0.05 as compared against sertraline + duloxetine treated group. ^P<0.05 as compared against sertraline + agomelatine.

Estimation of Serotonin Level:

Hippocampi: All drug treated groups showed statistically significant increase in serotonin level as compared to control group **Fig. 7**. Serotonin level was significantly increased in agomelatine (4 mg/kg) + duloxetine (5 mg/kg) treated group as compared to 'agomelatine alone' (8 mg/kg) and 'duloxetine alone' (10 mg/kg) treated group **Fig. 7**. Combination of sertraline (2.5 mg/kg) + duloxetine (5 mg/kg) treated group showed significant increase in serotonin levels, as compared to 'sertraline alone' (5 mg/kg) and 'duloxetine alone' (10 mg/kg) treated group **Fig. 7**. Combination of sertraline (2.5 mg/kg) + agomelatine (4 mg/kg) treated group showed significant increase in serotonin levels, as compared to 'sertraline alone' (5 mg/kg) and 'agomelatine alone' (8 mg/kg) treated group **Fig. 7**. Comparison of agomelatine (4 mg/kg) + duloxetine (5 mg/kg), sertraline (2.5 mg/kg) + duloxetine (5 mg/kg), sertraline (2.5 mg/kg) + agomelatine (4 mg/kg) treated group against three drug in combination *i.e.* agomelatine (2.67) + duloxetine (3.33 mg/kg) + sertraline (1.67 mg/kg) treated group showed significant increase in serotonin levels **Fig. 7**.

Cerebral Cortices: All drug treated groups showed statistically significant increase in serotonin level as compared to control group **Fig. 7**.

Combination treated group agomelatine (4 mg/kg) + duloxetine (5 mg/kg) showed significant increase in serotonin levels, as compared to 'agomelatine alone' (8 mg/kg) and 'duloxetine alone' (10 mg/kg) treated group **Fig. 7**. Combination of sertraline (2.5 mg/kg) + duloxetine (5 mg/kg) treated group showed significant increase in serotonin levels, as compared to 'sertraline alone' (5 mg/kg) and 'duloxetine alone' (10 mg/kg) treated group **Fig. 7**. Combination of sertraline (2.5 mg/kg) + agomelatine (4 mg/kg) treated group showed significant increase in serotonin levels, as compared to sertraline alone (5 mg/kg) and agomelatine alone (8 mg/kg) treated group **Fig. 7**. Serotonin levels was significantly increased in agomelatine (2.67 mg/kg) + duloxetine (3.33 mg/kg) + sertraline (1.67 mg/kg) treated group as compared to 'agomelatine alone (8 mg/kg)', 'duloxetine (10 mg/kg) alone' and 'sertraline (5 mg/kg) alone' and other combination treated group **Fig. 7**.

Whole Brain: All drug treated groups showed statistically increase in serotonin level as compared to control group **Fig. 7**. Combination treated groups such as agomelatine (4 mg/kg) + duloxetine (5 mg/kg) showed statistically increase in serotonin level as compared against 'agomelatine alone' (8 mg/kg) and 'duloxetine alone' (10 mg/kg) treated group. Sertraline (2.5 mg/kg) + duloxetine (5 mg/kg) treated group showed statistically increase in serotonin level as compared against 'sertraline alone' (5 mg/kg) and 'duloxetine alone' (10 mg/kg) treated group.

The combination of sertraline (2.5 mg/kg) + agomelatine (4 mg/kg) treated group showed statistically increase in serotonin level as compared against 'sertraline alone' (5 mg/kg) and 'agomelatine alone' (8 mg/kg) treated group. Serotonin levels was significantly increased in agomelatine (2.67 mg/kg) + duloxetine (3.33 mg/kg) + sertraline (1.67 mg/kg) treated group as compared to 'agomelatine alone (8 mg/kg)', 'duloxetine (10 mg/kg) alone' and 'sertraline (5 mg/kg) alone' and other combination treated group **Fig. 7**.

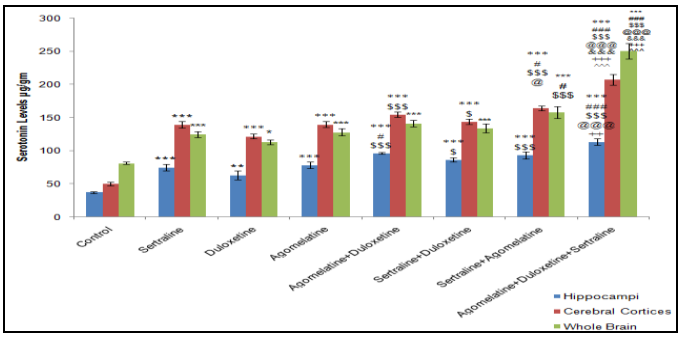

FIG. 7: ESTIMATION OF SEROTONIN LEVELS IN HIPPOCAMPI, CEREBRAL CORTICES AND WHOLE BRAIN BY HPLC-FD

Significant difference is denoted by ***P<0.001, **P<0.01, *P<0.05 as compared against control group. ###P<0.001, #P<0.05 as compared against sertraline treated group. $$$P<0.001, $P<0.05 as compared against duloxetine treated group. @@@P<0.001, @P<0.05 as compared against agomelatine treated group. &&&P<0.001, &&P<0.01, &P<0.05 as compared against agomelatine + duloxetine treated group. +++P<0.001, ++P<0.01 as compared against sertraline + duloxetine treated group. ^^^P<0.001 as compared against sertraline + agomelatine.

TABLE 7: ESTIMATION OF SEROTONIN LEVELS IN HIPPOCAMPI, CEREBRAL CORTICES AND WHOLE BRAIN BY HPLC-FD

Group no.	Treatment	Serotonin levels µg/gm of wet weight of tissue		
		Hippocampi	Cerebral cortices	Whole brain
1	Control	37.36 ± 1.39	250.20 ± 2.78	81.22 ± 1.58
2	Sertraline	74.68 ± 4.77***	138.99 ± 4.85***	124.64 ± 4.49***
3	Duloxetine	62.90 ± 6.88**	121.67 ± 3.73***	113.19 ± 3.64*
4	Agomelatine	78.01 ± 5.00***	139.60 ± 4.38***	127.98 ± 5.32***
5	Agomelatine + Duloxetine	95.96 ± 1.40***#$$$	154.68 ± 3.96***$$$	141.31 ± 4.87***
6	Sertraline + Duloxetine	86.49 ± 2.89***$	143.77 ± 3.70***$	133.83 ± 6.46***
7	Sertraline + Agomelatine	93.45 ± 5.35***$$$	163.99 ± 3.50***#$$$@	157.77 ± 9.10***#$$$
8	Agomelatine + Duloxetine + Sertraline	113.43 ± 4.68 ***###$$$@@@++	207.05 ± 8.24 ***###$$$@@@&&&++ +^^^	250.21 ± 11.78 ***###$$$@@@&& &+++^^^

Estimation of Norepinephrine Levels:

Hippocampi: All drug treated groups showed statistically significant increase in norepinephrine level as compared to control group **Fig. 8**. Agomelatine (4 mg/kg) + duloxetine (5 mg/kg) showed significant increase in norpinephrine level, as compared to 'agomelatine alone' (8 mg/kg) and 'duloxetine alone' (10 mg/kg) treated group **Fig. 8**.

Norepinephrine levels was significantly increased in sertraline (2.5 mg/kg) + duloxetine (5 mg/kg) treated group as compared to 'sertraline alone' (5 mg/kg) and 'duloxetine alone' (10 mg/kg) treated group **Fig. 8**. Combination of sertraline (2.5 mg/kg) + agomelatine (4 mg/kg) treated group showed significant increased in norepinephrine level as compared to 'sertraline alone' (5 mg/kg) and 'agomelatine alone' (8 mg/kg). Norepinephrine level was significantly increased in three drug combination treated group *i.e.* Agomelatine (2.67 mg/kg) + duloxetine (3.33 mg/kg) + sertraline (1.67 mg/kg) as compared to 'agomelatine alone' (8 mg/kg), 'duloxetine alone' (10 mg/kg) and 'sertraline alone' (5 mg/kg) treated group and other combination treated group.

Cerebral Cortices: All drug treated groups showed statistically significant increase in norepinephrine level as compared to control group **Fig. 8**. Agomelatine (4 mg/kg) + duloxetine (5 mg/kg) showed significant increase in nor-epinephrine level, as compared to 'agomelatine alone' (8 mg/kg) and 'duloxetine alone' (10 mg/kg) treated group **Fig. 8**. Norepinephrine levels was significantly increased in sertraline (2.5 mg/kg) + duloxetine (5 mg/kg) treated group as compared to 'sertraline alone' (5 mg/kg) and 'duloxetine alone' (10 mg/kg) treated group **Fig. 8**. Combination of sertraline (2.5 mg/kg) + agomelatine (4 mg/kg) treated group showed significant increased in norepinephrine level as compared to 'sertraline alone' (5 mg/kg) and 'agomelatine alone' (8 mg/kg). Norepinephrine level was significantly increased in three drug combination treated group *i.e.* Agomelatine (2.67 mg/kg) + duloxetine (3.33 mg/kg) + sertraline (1.67 mg/kg) as compared to 'agomelatine alone' (8 mg/kg), 'duloxetine alone' (10 mg/kg) and 'sertraline alone' (5 mg/kg) treated group and other combination treated group **Fig. 8**.

Whole Brain: All drug treated groups showed statistically significant increase in norepinephrine level as compared to control group **Fig. 8**. Agomelatine (4 mg/kg) + duloxetine (5 mg/kg) showed significant increase in norpinephrine level, as compared to 'agomelatine alone' (8 mg/kg) and 'duloxetine alone' (10 mg/kg) treated group **Fig. 8**. Norepinephrine levels was significantly increased in sertraline (2.5 mg/kg) + duloxetine (5 mg/kg) treated group as compared to 'sertraline alone' (5 mg/kg) and 'duloxetine alone' (10 mg/kg) treated group **Fig. 8**. Combination of sertraline (2.5 mg/kg) + agomelatine (4 mg/kg) treated group showed significant increased in norepinephrine level as compared to 'sertraline alone' (5 mg/kg) and 'agomelatine alone' (8 mg/kg) treated group **Fig. 8**. However, Norepinephrine level was significantly increased in three drug combination treated group *i.e.* Agomelatine (2.67 mg/kg) + duloxetine (3.33 mg/kg) + sertraline (1.67 mg/kg) as compared to 'agomelatine alone' (8 mg/kg), 'duloxetine alone' (10 mg/kg) and 'sertraline alone' (5 mg/kg) treated group and other combination treated group **Fig. 8**.

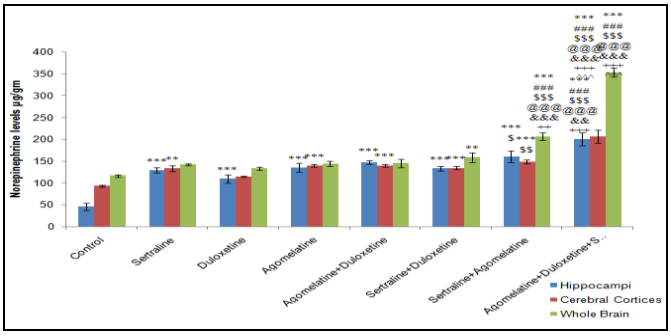

FIG. 8: ESTIMATION OF NOREPINEPHRINE LEVELS IN HIPPOCAMPI, CEREBRAL CORTICES AND WHOLE BRAIN BY HPLC-FD

TABLE 8: ESTIMATION OF NOREPINEPHRINE LEVELS IN HIPPOCAMPI, CEREBRAL CORTICES AND WHOLE BRAIN BY HPLC-FD

Group no.	Treatment	Norepinephrine Levels µg/gm of Wet Weight of Tissue		
		Hippocampi	Cerebral Cortices	Whole Brain
1	Control	45.60 ± 8.03	92.87 ± 2.81	116.43 ± 2.97
2	Sertraline	129.10 ± 5.5	133.18 ± 6.82**	141.55 ± 2.15
3	Duloxetine	108.97 ± 9.70	114.38 ± 1.65	132.92 ± 4.09
4	Agomelatine	134.81 ± 10.36	138.85 ± 3.01***	143.30 ± 5.92
5	Agomelatine + Duloxetine	146.91 ± 4.06	139.88 ± 2.57***	144.48 ± 9.87
6	Sertraline + Duloxetine	132.99 ± 4.85**	134.03 ± 3.86***	157.87 ± 10.76**
7	Sertraline + Agomelatine	160.69 ± 13.36***$	148.41 ± 3.77***$$	205.99 ± 8.547***###$$$@@@ &&&++
8	Agomelatine + Duloxetine + Sertraline	200.44 ± 15.30***###$$$ @@@&&+++	206.08 ± 14.54***###$$$ @@@&&&+++^^^	353.13 ± 10.74***###$$$@@@ &&&+++^^^

DISCUSSION: The purpose of this study was to evaluate the stress induced depression by antidepressant-like effect of sertraline, duloxetine and agomelatine by an animal model for depression and brain monoamine estimation by HPLC-FD.

Forced swim test (FST) on mice was employed. The immobility displayed by rodents when subjected to an unavoidable stress such as forced swimming is thought to reflect state of despair or lowered mood, which reflects depressive illness in humans. Additionally, immobility time has been shown to be reduced by treatment with antidepressant drugs [18]. The FST was designed to detect potential antidepressant compounds based on the abilities of clinically effective antidepressants to reduce the immobility in FST [18]. Though the FST does not adequately reflect the symptomalogy of human depression, it appears to have a higher predictive validity compared to other animal models additionally; it is sufficiently specific, since it discriminates antidepressants from neuroleptics and anxiolytics [2, 23]. The reduction in immobility period observed in forced swim and tail suspension tests with sertraline alone, agomelatine alone, and duloxetine alone treated groups is in agreement with previous reports [3]. The immobility period was significantly reduced in all monotherapy and combination treated groups, as compared to control groups. The reduction in immobility period was better with agomelatine plus duloxetine plus sertraline among combination treated groups.

Tail suspension test is widely accepted behavioural model for assessing pharmacological antidepressant activity [18, 21]. The antidepressant activity is expressed in terms of immobility period produced due to inescapable condition in tail suspension test, reflecting behavioural despair as seen in human depression [18]. False positive results to acute drug response, psycho stimulants, and varying sensitivity for genetic variations are the limitations of these models. These tests are more selective for monoamine-based mechanism analysis and have advantages like being the most predictive and widely used antidepressant models for screening antidepressant activity [7, 18]. The antidepressant effects of three drug in combination *i.e.* agomelatine, duloxetine and sertraline was explored using tail suspension test. All monotherapy and combination treated groups significantly reduced the immobility period as compared to control treated group. The reduction in immobility period was greater with agomelatine plus duloxetine plus sertraline among combination treated groups.

In addition to using behavioural animal models, a chronic stressor model 'Sucrose preference test' to test the antidepressant effect of drug treatments and combinations was also employed [8, 23]. In this model, mice were exposed sequentially to a variety of stressors. The procedure has been shown to produce several behavioral and hormonal disturbances which parallel to a large extent those found in depressed humans. The core symptom is a reduction of sucrose intake, a symptom equals to anhedonia [8] one of the core symptoms of depression as defined in the DSM-IV [1]. This paradigm has the advantage of excluding false positive effects caused by psycho stimulant agents in acute stress-based models. In spite of some initial disputes, the validity of sucrose preference test has been confirmed by numerous independent studies. Amount of sucrose consumed on 7^{th} day did not produce statistically significant results whereas 14 day treatment showed significant change in amount of sucrose consumed. The increase in sucrose consumption observed with agomelatine alone and duloxetine alone treated groups is in agreement with previous reports [10, 17]. Sertraline, agomelatine and duloxetine alone treated groups showed increased sucrose consumption as compared to the control group whereas duloxetine treated group showed less significant as compared to agomelatine and sertraline. Amongst the combination therapies agomelatine plus duloxetine and sertraline plus duloxetine and sertraline plus agomelatine showed significant increase in sucrose consumption as compared to respective monotherapies. The three drug combination, *i.e.* agomelatine plus duloxetine plus sertraline showed significant increase in sucrose consumption as compared to respective combination group and monotherapies.

In vitro estimation of dopamine level in the hippocampi and cerebral cortices, agomelatine showed significant elevated dopamine level as compared to control treated group whereas sertraline and duloxetine showed no significant change of dopamine level as compared to control

treated group. In combination therapies agomelatine plus duloxetine and sertraline plus duloxetine and sertraline plus agomelatine showed significant elevated dopamine level as compared to the respective monotherapy groups. While agomelatine plus duloxetine showed increase level of dopamine as compared to agomelatine alone and duloxetine alone treated group. In whole brain sertraline, duloxetine and agomelatine alone showed elevated levels of dopamine as compared to control treated group. Amongst the combination therapies sertraline plus agomelatine showed significant elevated dopamine level as compared to respective monotherapy groups and other combination treated group. In three drug combination therapies agomelatine plus duloxetine plus sertraline showed significant elevated dopamine level as compared to the respective monotherapies and respective combination treated group.

In vitro estimation of serotonin level in the hippocampi and cerebral cortices. In combination therapies agomelatine plus duloxetine and sertraline plus duloxetine and sertraline plus agomelatine showed significant elevated serotonin level as compared to the respective monotherapies while agomelatine plus duloxetine showed increase level of serotonin as compared to 'agomelatine alone' treated group and 'duloxetine alone' treated group. In hippocampi, cerebral cortices and whole brain sertraline, duloxetine and agomelatine alone showed elevated levels of serotonin as compared to control treated group. Amongst the combination group, sertraline plus agomelatine and agomelatine plus duloxetine plus sertraline showed significant elevated serotonin level as compared to respective monotherapies. In three drug combination therapies *i.e.* Agomelatine plus duloxetine plus sertraline showed significant elevated serotonin level as compared to the respective monotherapies and respective combination therapies.

In vitro estimation of norepinephrine level in the cerebral cortices, agomelatine and sertraline showed significant elevated norepinephrine level as compared to control treated group. Duloxetine showed no significant change as compared to sertraline treated group. In case of combination therapies, agomelatine plus duloxetine showed elevated norepinephrine levels as compared to the respective monotherapies. Sertraline plus duloxetine and sertraline plus agomelatine showed significant elevated norepinephrine level as compared to sertraline, duloxetine and agomelatine alone treated groups respectively. In cerebral cortices agomelatine showed significant change as compared to control treated group. In the combination therapies agomelatine plus duloxetine and sertraline plus duloxetine and sertraline plus agomelatine showed increased level of norepinephrine as compared to agomelatine, duloxetine and sertraline alone treated group respectively. Whereas sertraline plus agomelatine showed significant increase in norepinephrine level as compared to sertraline and agomelatine alone treated group.

In whole brain, sertraline plus agomelatine treated groups showed significant increased in nor-epinephrine level as compared to control group. Amongst the combination group, Sertraline plus agomelatine treated group and agomelatine plus duloxetine plus sertraline showed elevated level of norepinephrine as compared to the respective monotherapies. Agomelatine plus duloxetine plus sertraline showed significant elevation in the norepinephrine levels as compared to agomelatine, duloxetine and sertraline alone treated group and respective other combination treated groups.

Reports analyzing the effect of agomelatine treatment on dopamine and serotonin in rat prefrontal cortex have shown significant increase in dopamine levels. The present study findings are in agreement with the reported findings [6, 15]. The increase in dopamine levels was recorded not only in cerebral cortices but also in hippocampi and whole brain [6, 15]. Combination treated groups showed better serotonin and dopamine profile as compared to individual monotherapy. This may be due to combination of drugs having different mechanisms of action. About 90% of agomelatine is metabolized by CYP450 1A2 and about 10% by CYP450 2C9 isoforms. Agomelatine may increase the retention of drugs that are metabolized by CYP450 1A2, CYP450 2C9 and not by CYP1A1 or CYP450 2B6 [6]. SSRIs vary widely in their qualitative and quantitative interaction with cytochrome P450 (CYP) isozymes in the liver. CYP2D6 is inhibited by SSRIs. Sertraline is a naphthalenamine derivative with the predominant

pharmacological action of inhibiting presynaptic reuptake of serotonin from the synaptic cleft [5]. Sertraline inhibits serotonin uptake by rat brain synaptosomal preparations. Reports analyzing the effect of duloxetine treatment on monoamines in rat frontal cortex have shown significant increase in norepinephrine, dopamine and serotonin levels [11, 16].

The present study findings are in agreement with the reported findings [11, 16]. The increase in dopamine, norepinephrine and serotonin level not only in cerebral cortices but also in hippocampi and whole brain. Duloxetine metabolism involves CYP2D6 and CYP1A2 [14]. Duloxetine may increase the retention of drugs that are metabolized by CYP2D6 and not by CYP1A212. The different mechanism of action of agomelatine, duloxetine and sertraline may produce synergic effects rather than simply additive effects.

Overall the three drugs in combination treated groups was better in elevating dopamine, serotonin and norepinephrine in hippocampi, cerebral cortices and whole brain reducing the immobility period, increasing swimming and climbing count and increasing sucrose consumption among the combination treated groups.

CONCLUSION:

- Immobility was reduced in both forced swim and tail suspension test by agomelatine, duloxetine and sertraline and all combination treatment groups. Agomelatine plus duloxetine plus sertraline was better among combination approaches in terms of reduction in immobility time period.

- In sucrose preference test, agomelatine plus duloxetine plus sertraline was better among monotherapies and combination treated group.

- Agomelatine plus duloxetine, sertraline plus duloxetine, sertraline plus agomelatine and agomelatine plus duloxetine plus sertraline elevated all 3 brain monoamines *viz*; dopamine, serotonin and norepinephrine in hippocampi, cerebral cortices and whole brain. Agomelatine plus duloxetine plus sertraline showed better brain monoamine profile as compared to other combination groups.

- Overall, consideration of these augmentation approaches at half of their respective monotherapy doses may result in decreased prevalence of associated side / adverse effect. These findings can be further probed in a clinical setting as a future endeavour.

- Thus, stress induced depression was manage by using forced swim test, tail suspension test and sucrose preference test. Agomelatine plus duloxetine plus sertraline showed slightly better brain monoamine profile by increasing dopamine, serotonin, norepinephrine level in hippocampi, cerebral cortex and whole brain and decrease in immobility period, increase in swimming count and climbing count and increase sucrose consumption than other treated group.

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CONFLICT OF INTEREST: The authors declare that there is no conflict of interest.

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