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EVIDENCE FOR THE ROLE OF NOR-ADRENERGIC AND SEROTONERGIC SYSTEMS BUT NOT THE OPIOID SYSTEM IN THE ANTIDEPRESSANT ACTIVITY OF AQUEOUS EXTRACT OF *ABELMOSCHUS ESCULENTUS* SEEDS IN THE MOUSE FORCED SWIM TEST

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Keywords:

AEAES, Serotonergic, Nor-adrenergic, Anti-depressant like activity, Albino female mice, Forced swim test

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ABSTRACT: Background: Depression is a state of mental illness characterized by long-lasting moods of desolation. Typical to atypical antidepressants are available in modern medicine for depression therapy, but the side effects are still questionable. Aqueous extract of *Abelmoschus esculentus* seeds *(AEAES)* from the Okra has been chosen for the study. *AEAES* has already been shown to exhibit an antidepressant-like effect, but there is no information about its mechanism. The current study has studied the monoaminergic and opioid systems' involvement in the antidepressant effect of *AEAES*. **Methods:** The current study has shown the signal for the role of nor-adrenergic and serotonergic systems in the antidepressant activity of *AEAES* in mice using the Forced swim test (FST). Swiss Albino female mice were treated with vehicle *AEAES* (200 mg/kg) orally, and different types of receptor antagonists were used to demonstrate the mechanism of action of an *AEAES*. **Results:** The antidepressant action exhibited by *AEAES* was obliterated by the pre-treatment of mice with p-chlorophenylalanine (100 mg/kg, i.p., serotonin synthesis inhibitor), Ketanserin (5 mg/kg, i.p., a $5HT_{2A/2B}$ antagonist) and Prazosin (1 mg/kg, i.p., an $\alpha_1$blocker) but the effect was not abolished with Pindolol (10 mg/kg, i.p., $5HT_{1A/1B}$ antagonist), Ondansetron (1 mg/kg, i.p., $5HT_3$antagonist), Yohimbine (1 mg/kg, i.p., an α_2 blocker) and Naloxone (1 mg/kg, i.p., an opioid antagonist). **Conclusions:** The current study delivers evidence that the antidepressant activity of *AEAES* in FST is mediated through the interaction with the serotonergic receptor ($5\text{-}HT_{2A/2B}$) and noradrenergic receptor (α-1 adrenoceptor) without influencing the opioid receptors.

INTRODUCTION: Depression, the most common affective or mood disorder along with mania, is considered a devastating disorder with disturbing mood.

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It is an extremely prevalent and disabling illness [1]. It is a state of mental illness characterized by profound, long-lasting moods of desolation or anguish that can modify one's feelings, behaviour, and sense of well-being in the worst manner.

Depression can be a major or a minor type associated with hallucinations and delusions in a severe form of illness. Although counselling and psychotherapy are considered the better therapeutic option for mild to moderate depression, pharmacotherapy plays a foremost part in treating

depression in moderate to severe stages and preventing its complications such as suicidal thoughts [2]. Various classes of drugs, from typical to atypical antidepressants, are available today in modern medicine for the therapy of depression. Though these drugs help to alleviate the depressive symptoms, the side effects are still questionable [3]. Hence, it is the need of the hour to look for new targets such as herbal extracts used not only to lessen the depressive symptoms but with relatively less or nil side effects. Aqueous extract of *Abelmoschus esculentus (AEAES)* is one of such herbal extract from the Okra or Ladies finger seeds which have shown to exhibit antidepressant activity after both acute and chronic administration in animal models of depression [4].

Though the antidepressant-like effect had been shown for *AEAES*, none of the studies have explained the possible mechanism of action of *AEAES* as antidepressant so far, to my knowledge. 'Monoamine hypotheses' stated that monoamine neurotransmitters such as Serotonin and Nor-adrenaline deficiency at the synapse remain a main reason for the development of depressive symptoms. Apart from the monoaminergic system, the opioid system is also activated by some classical antidepressants to reduce the symptoms of depression. Hence it is necessary not only to investigate the role of monoaminergic system in the antidepressant activity of *AEAES* but also the role of the opioid system in it [5, 6]. Therefore, to explore the mechanism of action of *AEAES* as an antidepressant, the current study was conducted to investigate the possible role of monoaminergic and opioid systems in the antidepressant activity of *AEAES* by using behavioural models of depression in mice.

MATERIALS AND METHODS:

Study Design: An animal interventional study was conducted to demonstrate how an extract *AEAES* exhibits the antidepressant-like effect, *i.e*., its possible mechanism of action in albino mice.

Animals: 2-4 months old Female Swiss albino adult mice with 25-30 grams were used. Mice were maintained at 22–25°C with 12:12 hr cycle with access to food and water in a polypropylene cage in groups with 6 mice in each group. Experimental procedures were conducted between 9 am to 4 pm on a daily basis. Institutional Animal Ethics Committee (IAEC) approval was obtained (IAEC approval no.: PIMS/13-IAEC/N-15/2016), and procedures were conducted according to CPCSEA guidelines.

Drugs: The test drug (Herbal extract) *AEAES* was obtained from CL BaidMetha College of Pharmacy, Chennai, India. Phytochemical analysis of *AEAES* showed the presence of alkaloids, carbohydrates, quercetin, rutin, phenols, proteins, terpenoids, tannins, and sterols.

The extract was reconstituted in distilled water and then fed to the animals. Drugs used: Ketanserin (5mg/kg i.p), Pindolol (10mg/kg i.p), Ondansetron (1mg/kg i.p), Naloxone (1mg/kg i.p), Yohimbine - 1mg/kg i.pwere obtained from Sigma Aldrich chemicals private limited; Prazosin (1mg/kg i.p, Sun Pharmaceuticals Ltd., India); Chemicals used: pCPA (parachlorophenylalanine) - 100mg/kg i.p (Sigma Aldrich chemicals private limited);Control-Distilled water.

Methods:

Experimental Procedure: The experiment was conducted in the Experimental pharmacology lab, Pharmacology department, PIMS. Appropriate drug doses were calculated according to the weight of the mice, and then the drugs were administered to each mouse in different groups. All the tests were performed after 1 hour of administering the test drug orally (p.o) and after 30 minutes of administration of blocker intraperitoneally (i.p) [5]. A total of 84 Swiss Albino female mice were used to investigate the possible mechanism of action of *AEAES* by dividing it into 14 groups with 6 animals in each group.

For Evaluation of Possible Mechanism of Anti-depressant Action: The dose for the test drug *AEAES* was taken from the earlier study where the evaluation of the antidepressant-like effect of *AEAES* using three different doses (100mg, 200mg, and 400mg) was performed [4]. Among these three doses, a single effective dose (*AEAES*: 200 mg/kg) which had produced significant antidepressant action was selected and used for evaluating possible mechanisms of antidepressant action using the Forced swim test (FST) [4, 5]. The doses, routes of administration, and pre-treatment time interval of

the antagonists were also selected from the previous studies [6, 7, 8]. Animals were initially treated with the specific receptor antagonists pertaining to the system to be evaluated or the distilled water, following which *AEAES* was administered at a dose of 200 mg/kg, p.o. after 30 minutes. FST was performed after 1 hour of *AEAES* administration.

Serotonergic System Involvement in the Antidepressant Activity of *AEAES* using FST: A pre-treatment with pCPA injection (serotonin synthesis inhibitor) at a 100 mg/kg dose is administered intraperitoneally once a day for four days continuously to the mice.

Following half an hour of administration of pCPA injection on the fourth day, animals were given either distilled water or *AEAES*, and FST was performed after 1 hour [9]. The possible role of 5-HT1A/1B, 5-$HT_{2A/2C}$ and 5-HT_3 receptors in the antidepressant-like activity of AEAES was further evaluated using specific receptor blockers. Therefore, mice were pre-treated with Pindolol (10 mg/kg, i.p., a 5-$HT_{1A/1B}$ receptor antagonist), Ketanserin (5 mg/kg i.p., a $5HT_{2A/2C}$ receptor antagonist) or Ondansetron (1 mg/kg i.p., $5HT_3$ receptor antagonist). After half an hour following treatment with antagonists, they received *AEAES* or distilled water, and FST was performed after 1 hour [5, 6, 9].

Nor-adrenergic System Involvement in the Antidepressant Activity of *AEAES* in FST: Mice were pretreated with an α_1-adrenergic blocker, Prazosin at a dose of 1 mg/kg i.p., or an α_2-adrenergic blocker, Yohimbine at a dose of 1 mg/kg administered intraperitoneally. They received *AEAES* or distilled water after 30 min of antagonist administration, and FST was done after 1 hour [6, 9, 10].

Opioid system involvement in the antidepressant activity of *AEAES* using FST: Mice were pretreated with a non-selective opioid receptor antagonist Naloxone at a dose of 1mg/kg, intraperitoneally. They received *AEAES* or distilled water after 30 minutes of antagonist administration, and FST was done after 1 hour [6, 11].

Behavioural analysis was done using FST since it delivers a rapid and reliable behaviour screening test for antidepressants, and they are extensively used, models.

Forced Swim Test (FST): Immobility duration in the FST was calculated to assess the antidepressant property of the test drug. Briefly, 60 min after oral administration of a drug, each mouse was allowed to swim one by one in the 5-liter beaker with water filled up to 15 cm of height, and the immobility period was noted for the last 4 min of a 6 min test.

When a mouse floated without any movement or tried to put its head above the surface of the water, it was considered that the mouse was immobile [12, 13].

Statistical Analysis: The Entire parameters noted in the current study were tabularized and presented as mean ± standard error of the mean. One-way ANOVA (analysis of variance) followed by posthoc Bonferroni correction was used to analyze data amongst different groups.

For all inferential statistical tests, a $P < 0.05$ was considered statistically significant, and a $P < 0.01$ was considered extremely statistically significant. GraphPad in Stat software of version 3.06 was used to analyze data.

RESULTS: Following the result published in the previous study that "*AEAES* produced significant antidepressant-like effect at a dose of 200 mg/kg", we did a study to demonstrate the possible mechanism of action of the *AEAES*.

The Nor-adrenergic System Involvement in the Antidepressant Activity of *AEAES* in FST: Prazosin or Yohimbine, the alpha-adrenergic blockers were administered 30 min before administering *AEAES* and FST was done following 1 hour after *AEAES* administration.

The antidepressant activity of *AEAES* (200mg/kg, p.o.) was pointedly reversed by treatment of mice priorly with Prazosin, an alpha-1 blocker (1mg/kg, i.p.) but not with Yohimbine, an alpha-2 blocker (1 mg/kg, i.p.) **Table 1** and **Fig. 1**. ANOVA followed by the Post hoc Bonferroni test showed a significant effect of *AEAES*-Prazosin interaction with $P<0.001$. On the other hand, the pre-treatment with Yohimbine did not show any significant activity.

TABLE 1: EFFECT OF AEAES PRE-TREATED WITH ALPHA NOR-ADRENERGIC BLOCKERS (α1 AND α2 RECEPTORS) ON IMMOBILITY PERIOD OF MICE USING FORCED SWIM TEST

Groups	Immobility duration (seconds)
Control (Distilled water)	136.54±2.04
AEAES 200mg/kg	60.80±2.21
Prazosin (1mg/kg)	102.33±2.07
Prazosin +AEAES 200mg/kg	109.13±2.97***
Yohimbine (1mg/kg)	106.21±3.91
Yohimbine +AEAES 200mg/kg	71.00±3.40^{NS}

n = 6 in each group; Data expressed as Mean±SEM was analyzed by one-way, ANOVA followed by Post-hoc Bonferroni test;*** denotes p<0.001 when compared with the control group; ^{NS} – Not significant

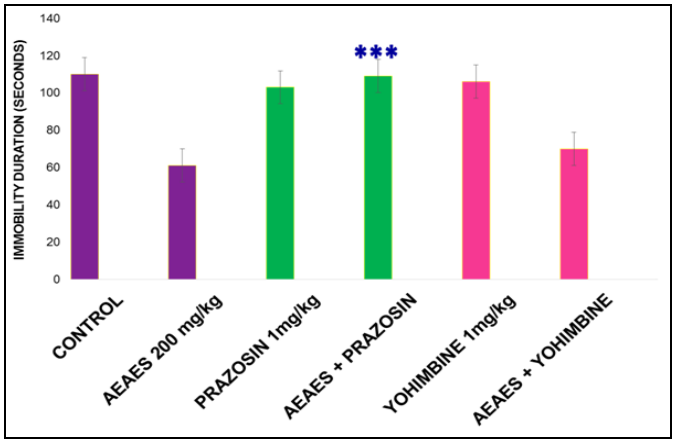

FIG. 1: EFFECT OF *AEAES* PRE-TREATED WITH ALPHA NOR-ADRENERGIC BLOCKER (α_1 AND α_2 RECEPTORS) ON IMMOBILITY PERIOD OF MICE USING FORCED SWIM TEST. n = 6 in each group; Data expressed as Mean±SEM was analyzed by one-way, ANOVA followed by Post-hoc Bonferroni test;*** denotes p<0.001 when compared with the control group; NS – Not significant

The Serotonergic System Involvement in the Antidepressant Activity of *AEAES* in FST: The Serotonergic blockers namelypCPA, Pindolol, Ketanserinor Ondansetron, were administered 30 min before *AEAES* and following 1 hour after *AEAES* administration, FST was done. The antidepressant-like effect of *AEAES* (200 mg/kg, p.o.) was reversed by pre-treatment of mice with pCPA, a serotonin synthesis inhibitor (100 mg/kg, i.p.), and Ketanserin, a $5HT_{2A/2B}$ receptor antagonist (5 mg/kg, i.p.) but not with Pindolol and Ondansetron **Table 1** and **Fig. 1**. ANOVA followed by the Post hoc Bonferroni test showed a significant effect of *AEAES*-pCPA interaction and *AEAES*-Ketanserin interaction with P<0.001. But on the other hand, the pre-treatment with Pindolol and Ondansetron did not show any significant activity.

TABLE 2: EFFECT OF AEAES PRETREATED WITH SEROTONERGIC SYNTHESIS INHIBITOR & BLOCKERS ($5\text{-}HT_{2A/2C}$, $5\text{-}HT_3$ AND $5\text{-}HT_{1A/1C}$ RECEPTORS) ON IMMOBILITY PERIOD OF MICE USING FORCED SWIM TEST

Groups	Immobility duration (seconds)
Control (Distilled water)	136.54±2.04
AEAES 200mg/kg	60.80±2.21
pCPA(100mg/kg)	103.23±2.56
pCPA + AEAES	101.50±2.80***
Ketanserin 5mg/kg	101.66±3.27
Ketanserin+ AEAES	111.16±2.59***
Ondansetron 1mg/kg	116.73±3.52
Ondansetron + AEAES	78.83±2.44^{NS}
Pindolol 10mg/kg	111.01±2.80
Pindolol + AEAES	61.66±1.66^{NS}

n = 6 in each group; Data expressed as Mean±SEM was analyzed by one-way ANOVA followed by Post-hoc Bonferroni test; *** denotes p<0.001 when compared with the control group; ^{NS} – Not significant.

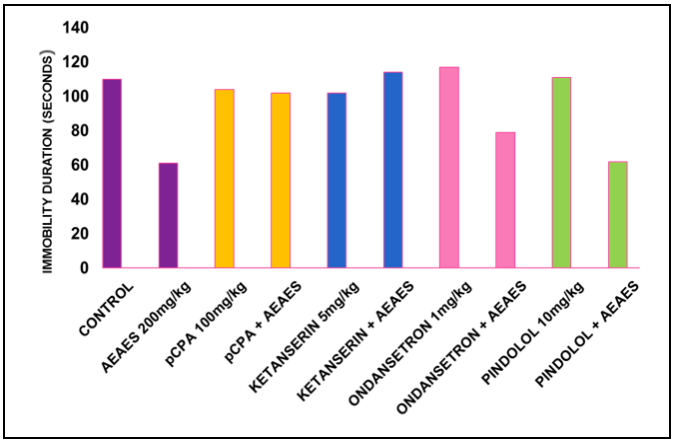

FIG. 2: EFFECT OF AEAES PRETREATED WITH SEROTONERGIC SYNTHESIS INHIBITOR & BLOCKERS ($5\text{-}HT_{2A/2C}$, 5-HT3 AND $5\text{-}HT_{1A/1C}$ RECEPTORS) ON IMMOBILITY PERIOD OF MICE USING FST. N (Number of mice) = 6 in each group; Data expressed as Mean±SEM was analysed by one-way ANOVA followed by Post-hoc Bonferroni test**;** *** denotes p<0.001 when compared with control group; ^{NS} – Not significant

The Opioid System Involvement in the Antidepressant Activity of *AEAES* in FST: Naloxone, the opioid antagonist was administered 30 min prior to *AEAES* and following 1 hour after *AEAES* administration, FST was done. The antidepressant-like effect of *AEAES* (200 mg/kg, p.o.) was not reversed by pre-treatment of mice with Naloxone, opioid receptor antagonist (1 mg/kg, i.p.) **Table 3** and **Fig. 3.** ANOVA followed by Post hoc Bonferroni test did not show a significant activity of AEAES-Naloxone interaction.

TABLE 3: EFFECT OF AEAES PRE-TREATED WITH OPIOID RECEPTOR BLOCKER (μ-RECEPTOR) ON IMMOBILITY PERIOD OF MICE USING FORCED SWIM TEST

Groups	Immobility Duration (seconds)
Control (Distilled water)	136.54±2.04
AEAES 200mg/kg	60.80±2.21
Naloxone 1mg/kg	102.66±1.99
Naloxone + AEAES	79.66±2.82^{NS}

N (Number of mice) = 6 in each group; Data expressed as Mean±SEM was analyzed by one-way ANOVA followed by Post-hoc Bonferroni test; ^{NS} – Not significant.

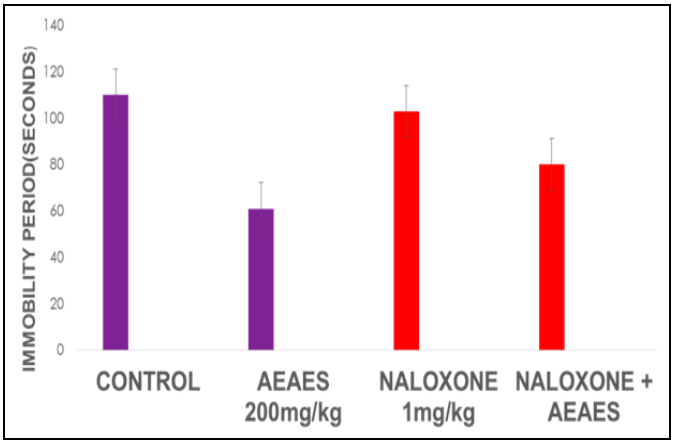

FIG. 3: EFFECT OF AEAES PRETREATED WITH OPIOID RECEPTOR BLOCKER (μ-RECEPTOR) ON IMMOBILITY PERIOD OF MICE USING FORCED SWIM TEST. N (Number of mice)= 6 in each group; Data expressed as Mean±SEM was analyzed by one-way ANOVA followed by Post-hoc Bonferroni test ^{NS} – Not significant.

DISCUSSION: Though both typical and atypical antidepressants reduce the symptoms and severity of depression, their drawbacks, such as side effects and slower onset of action, remain. Hence, we have chosen the herbal extract, aqueous extract of Abelmoschus esculentus seeds (AEAES), commonly known as seeds of Ladies finger as our study compound, and proved the anti-depressant-like effect of this extract in mice using animal models of depression [4]. But still, there is a lack of information about how this extract produces the antidepressant-like activity. Thus, to investigate the mechanism of action of *AEAES*, we have conducted the current study. To our searched data, this is the first study that established the mechanism of action of the *AEAES*. As we had discussed in the previous study, the active principle that is accountable for the antidepressant activity of *AEAES* are flavonoids, namely quercetin and rutin which has already been proved by the phytochemical analysis of the *Abelmoschus esculentus* extracts [4, 14]. The dose of *AEAES* (200 mg/kg) to proceed for mechanism evaluation was selected from the previous study. Among the different doses, we had chosen the 200 mg/kg since it produced a significant effect compared to other doses [1, 4]. Forced Swim Test was used as an animal model of behaviour to find out the difference in immobility duration since it is considered the more consistent and widely used animal model of depression [15]. We have used only female mice in our study so that dissimilarities that ensue due to variations in gender were lessened [16].

Dual studies had described that the pathophysiological basis of depression was due to a depleted level of monoamines and/or dopamine in the Central Nervous System. This hypothesis seems to be strengthened by the mechanism of antidepressant drugs, which raise all these neurotransmitter levels in the brain. And also stated that the FST affords a suitable model for exploring neurobiological mechanisms for antidepressant effects [17, 18]. Therefore we conducted the study to show the role of serotonergic, noradrenergic, and opioid systems on the effects of *AEAES*. Using this aim, the present study examined the effects of some pharmacological blockers/modulators of the above-mentioned systems on the anti-immobility action of *AEAES* in mice.

Various studies conducted to evaluate the Serotonergic system's role in the antidepressant activity of apigenin, curcumin and GMP (Guanosine Monophosphate) respectively reported that pCPA, serotonin synthesis inhibitor was able to destroy the endogenous serotonin effectively after giving pCPA for four days continuously without disturbing the noradrenergic or dopaminergic levels [9, 19, 20]. In the current study, the anti-immobility effect induced by *AEAES* in FST has been prevented by pre-treatment with pCPA, suggesting the serotonergic system involvement in the antidepressant activity of this extract. Mice were pretreated with different serotonin receptor antagonists like Ketanserin, which is the 5-$HT_{2A/2B}$ receptor blocker, Pindolol (5-$HT_{1A/1B}$ blocker), and $5HT_3$ receptor antagonist, Ondansetron. It was noticed that the anti-immobility activity formed by extracting the FST was obliterated by the treatment of mice priorly with Ketanserin, which is the 5-$HT_{2A/2B}$ receptor blocker. The 5-$HT_{1A/1B}$ blocker,

Pindolol and an antagonist of $5HT_3$ receptor, Ondansetron, could not obliterate the antidepressant activity exerted by *AEAES*. These outcomes specify that the antidepressant activity of *AEAES* involves the $5HT_2$ serotonergic receptors interaction but not $5HT_1$ and $5HT_3$ receptor interactions. Few studies reported the interaction of either an extract or flavonoids with serotonergic receptors in producing an antidepressant-like effect on the monoaminergic system, indicating a comparable mode of action of these compounds [6, 17, 21, 22].

Apart from the serotonergic system, the involvement of the noradrenergic system was reported by the study, which pointed out that antidepressants can act by augmenting NA levels in the synaptic clefts [23]. In this perspective, few other studies also emphasized that the α1 and α2 receptor blockers have been shown to cause some of the antidepressant-like effects of drugs in certain behavioural models of depression [24, 25]. In our study, the reduction in immobility duration caused by *AEAES* was inverted by pre-treatment of mice with an α-1 adrenoceptor blocker, Prazosin but not with an α-2 adrenoceptor blocker, Yohimbine signifying the involvement of only α-1 adrenoceptor in the antidepressant-like action of *AEAES* in the FST. These outcomes are comparable with the studies where it has been revealed that the antidepressant effect of compounds used in their study was found to be inverted only by α-1 blocker and not by α-2 blocker in animal models [26, 27].

Evidence for the role of opioid receptors in depression was proven in some studies where it has revealed that there is a marked drop in μ-opioid receptor levels in the patients with depression [8, 28, 29]. In the current study, Naloxone was unable to oppose the reduction in immobility time caused by *AEAES* in the FST, demonstrating that the antidepressant activity of *AEAES* was not facilitated through opioid receptors. Similar results were also observed in the study conducted by C. Girish *et al.*, who studied the role of the monoaminergic and opioid systems in the antidepressant activity by using two different extracts, namely ellagic acid and *Bacopa monnieri*in two different studies [5, 6]. Together, behavioural changes induced by *AEAES* in the FST suggested that an antidepressant activity appear to be facilitated by an interaction with the serotonergic and noradrenergic but not with the opioid systems. The present study specifies the role of the monoaminergic system in the antidepressant activity of *AEAES*. The precise mechanism by which *AEAES* modifies the monoaminergic system is somewhat uncertain. There may also be an involvement of other mechanisms, which are not mentioned in this work. The neuroprotective effect of *AEAES* is thought to be due to its antioxidant and antistress activities, which have been revealed in the study by Dordeulla *et al.* and Ebrahimzadesh *et al.* [1, 14].

A study described that a stress response is due to the activation of the hypothalamus-pituitary adrenal axis, causing a rise in blood corticosterone levels that in turn lead to an elevation in serum triglycerides levels and hyperglycemia [30, 31]. Another study showed that administration of *Abelmoschus esculentus* seed extracts (AE and ME) significantly opposed the acute restraint stress-induced raised blood glucose, corticosterone, triglycerides, and cholesterol levels in mice. This concept demonstrated stress-relieving property of Ladies' finger seeds [14]. Another study on *Abelmoschus esculentus* also supported the above findings that its fruit extract and its active principles (quercetin and rutin) protected neuronal function and enhanced learning and memory deficits produced by chronic treatment (21 days) of Dexamethasone (60mg/kg) by means of Morris water maze task [32].

Acetylcholinesterase (AChE) inhibition is still considered one of the principal therapeutic approaches against Alzheimer's disease (AD) and cognitive deficits. The study by Patel et al. and Szwajgier showed that various plant-derived phytochemicals claimed for nootropic activity has produced AChE inhibitory effect and/or antioxidant activity [33, 34]. Dordeulla *et al.* opined that there was no report on AChE inhibitory effect of *Abelmoschus esculentus* [14]. However, Szwajgier pointed out that its major bioactive principles, quercetin, and rutin, have been demonstrated for AChE inhibitory effect [34]. Keeping this in mind, further studies should be conducted to explore the precise mechanism involved in the antidepressant-like activity of aqueous extract *Abelmoschus esculentus* seeds.

CONCLUSION: To conclude, our study demonstrates the mechanism of antidepressant activity of *AEAES* in animal behaviour depression models. The current study delivers evidence that the antidepressant activity of *AEAES* in FST is mediated through the interaction with the serotonergic (5-$HT_{2A/2B}$) and noradrenergic (α-1 adrenoceptor) systems without influencing serotonergic receptor subtypes (5-$HT_{1A/1B}$ and 5-HT_3), noradrenergic (α-1 adrenoceptor) as well as the opioid system. These findings are in agreement with the studies conducted by Girish *et al.,* (2016), MCO Cito *et al.,* (2015), An *et al.,* (2008); Yi *et al.,* (2010), Tubuly *et al.,* (2008), Stone *et al.,* (2003) and C. Girish *et al.,* (2012). However, more experimental studies are needed to validate whether AEAES will produce a similar valuable effect in depressed individuals and infer the detailed mechanism of action at the cellular and molecular levels.

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