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Evaluation Of Antianxiety-Like Activity Of Astaxanthin And Its Possible Mechanism Of Action Using Animal Models.

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ABSTRACT

The incidence of anxiety rapidly increasing among community. Present treatments have many side effects. Astaxanthin is a xanthophyll carotenoid which is found in various microorganisms and marine animals. It's got antioxidant properties and neuroprotective effect. To evaluate the antianxiety-like activity of Astaxanthin and its possible mechanism of action using animal models namely elevated plus maze and hole board tests. Fifty-four healthy adult male swiss albino mice were used to study the acute and chronic antianxiety-like effect of Astaxanthin (1, 3 and 5 mg/kg p.o.). After a washout period of 1 month, all the groups were used to study the mechanism of action by blocking the Serotonergic and GABAergic system with the subsequent drugs namely p-Chlorophenylalanine (a serotonin synthesis inhibitor), pindolol ($5HT_{1A/1B}$ receptor antagonist), picrotoxin (a non-competitive GABA receptor antagonist) and flumazenil (a benzodiazepine site antagonist). The time spent within the open arm using elevated plus maze and the number of dipping's and squares crossed within the hole board test was recorded as mean±SEM and analysed by one-way ANOVA test with post-hoc Bonferroni correction. Our results showed that Astaxanthin when administered acutely or chronically to mice (3, 5 mg/kg, p.o), produced a significant anxiolytic effect ($p<0.05$) using both the behavioral models with a profile comparable to that of diazepam (1 mg/kg, p.o) mediated via serotonergic systems.

Keywords: Astaxanthin, Anxiety, Serotonin, Diazepam

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INTRODUCTION

Anxiety is one among the foremost common psychiatric disorder in children and adolescents[1]. These disorders, are the most common cause for various psychological state and regularly cause significant functional impairment. This psychological problem negatively impacts the functioning of routine activities and increases the danger of other sorts of disorders.[2] A recent epidemiological study commissioned by the Canters for Disease Control found that 3.0% of children in the United States aged 3–17 years had current anxiety diagnoses during the years 2005–2011. [3] Anxiety disorders that occur in children and adolescents' period which forward the young people at risk for co morbid depressive disorders, drug dependence, suicidal behaviour, and educational underachievement.[1]The current treatment constitute mainly benzodiazepines and selective serotonin reuptake inhibitors (SSRIs), two classes of drugs that both have side effects. Benzodiazepines are related to ataxia, sedation, skeletal muscle relaxation, amnesia, and interactions with ethanol and barbiturates; whereas, SSRIs show a slow onset of the anxiolytic activity.[4] Thus, there is requirement of strong anxiolytic compounds that have lesser side effects and immediate onset of action.

Astaxanthin, a unique member of the xanthophylls, a deep red-colored phytonutrient that can be synthesized by a microalgae called *Haematococcus pluvialis*.[5] Astaxanthin could also be a strong antioxidant and antiinflammatory agent. It has a unique property of transmembrane location because of its polar and nonpolar structure. The Polar side has ion ring which is a strong free radical scavenger while the nonpolar transmembrane domain helps in moving the free radicals rapidly outside the cell membrane.[6]

Synaptic plasticity and neurogenesis are decreased with ageing. Astaxanthin stimulates BDNF (Brain-derived neurotrophic factor). ERK (extracellular signal-regulated kinases) is activated by BDNF, it can cause the transcription of genes needed for synaptic plasticity and neurogenesis which successively increases the memory.[7]

MATERIALS AND METHOD

The experiments were performed on healthy adult male swiss albino mice weighing between 20-30g.The experimental protocol was approved by the Institutional animal ethics committee and was executed according to the guidelines of the Committee for the Purpose of Control and Supervision for Experiments on Animals (CPCSEA) India. The animals were obtained from the animal house of king's research institute, Chennai. All efforts were made to minimize the suffering and to reduce the number of animals used in the experiments by following the code of ethics for animal experiments. Animals were housed 6 per cage at 24°C to 30°C under a 12 hours light, dark cycle and ad libitum access to water and food. Behavioral experiments were undertaken between 9:00am to 4pm.

All groups of mice were acclimatized in test environment 1 week before the test. Fifty- four animals were included in this study with 6 animals in each group for the Period of 4 months. All groups of male Albino mice will be given their respective drugs once daily between 9 AM and 12 AM. Acute study followed by chronic study (10 days test drug administration) for all the groups was performed by using elevated plus maze and hole board. The same Animals in control group and standard group are used for both elevated plus maze and hole board test. For understanding the mode of action of Astaxanthin the blockers are used against $GABA_A$ receptor and 5-$HT1_A/1_B$ receptor

Drugs

All the drugs used were purchased from standard commercial suppliers. Astaxanthin was obtained from zenith nutrition, Bangalore. picrotoxin ($GABA_A$ receptor antagonist), p-chlor- ophenylalanine (PCPA, inhibitor of serotonin synthesis) and pindolol (a β-adrenoceptors blocker/5-$HT1_A/1_B$ receptor antagonist), were obtained from Sigma Chemical co, USA. Flumazenil ($GABA_A$/benzodiazepine receptor antagonist) and diazepam (positive allosteric modulator of $GABA_A$ receptor) ampoules were diluted with 0.9% saline and administered to mice**.** Picrotoxin, PCPA and pindolol were dissolved in 0.9% saline

Experimental methods

Evaluation of the antianxiety like effect after acute treatment with Astaxanthin:

Animals received a single oral dose of saline, Astaxanthin, diazepam (1 mg/kg) and were tested for anxiolytic-like effect using the elevated plus-maze. Astaxanthin was mixed in saline and given at three different dose ranges 1mg/kg, 3mg/kg, 5mg/kg.[6] Therefore, we have figure out the anxiolytic-like activity 30 min after Astaxanthin administration. Diazepam (1 mg/kg, single oral dose) was administered 30 min before the tests and kept as a positive control. The dose which give anxiolytic activity was taken for mechanism of action.

Evaluation of the anxiolytic-like effect after chronic treatment with Astaxanthin:

For the chronic study, saline, diazepam (1 mg/kg) and Astaxanthin 1mg/kg, 3mg/kg, 5mg/kg were administered to mice for 10 days. The drugs were given as a single dose per day in between 9 am to 10 am. After 24 hr of the last dose, the animals were evaluated for anxiolytic-like effect using the elevated plus-maze.

Evaluation of Astaxanthin possible mechanism of action using elevated plus-maze

Evaluating the GABAergic system in the anxiolytic-like action of Astaxanthin using the elevated plus-maze.

Animals were pre-treated with picrotoxin (1 mg/kg, i.e., a $GABA_A$ receptor antagonist) and flumazenil (1 mg/kg, i.e., a GABAA/benzodiazepine receptor antagonist) 30 min before they received Astaxanthin or vehicle and were tested on the elevated plus-maze after 30 min.

Role of the serotonergic system in the anxiolytic-like effect of Astaxanthin using the elevated plus-maze.

A group of mice received an injection of PCPA (100 mg/kg i.p., a serotonin synthesis inhibitor) once daily for four consecutive days as pretreatment. 30 min after the last injection of PCPA, mice were treated with either a vehicle or Astaxanthin and tested on the elevated plus- maze. Further, to investigate the possible involvement of 5-$HT1_A/1_B$ receptor in the antianxiety-like effect of Astaxanthin, animals were pretreated with pindolol (10 mg/kg, i.p,)

Behavioural analysis

Elevated Plus Maze Test: The elevated plus maze is commonly used to assess anxiety related behaviour. In the elevated plus maze, avoidance of the open arms, an increase in the time spent in the closed arms and a decrease in rearing indicates anxiety. In the elevated plus maze model, the number of entries and the preference for open arms by astaxanthin treated rats was significantly increased ($P< 0.05$) as compared to control. The number of entries in the closed arm by astaxanthin treated rats significantly decreased ($P<0.05$) as compared to control.[8]

Hole Board test: Head dipping behaviour of the animals in the Hole Board test is considered to be an indicator of anxiety. Increased frequency and duration of the head dipping is an indicator of anxiolytic state. [6] As a positive standard, Diazepam significantly increases both the frequency and duration of head dipping ($p<0.01$) but in comparison with astaxanthin (3mg,5mg/kg/day) administered along with the standard drug, it produced an even more significant antianxiety effect ($p <0.01$).

RESULTS

The effect of acute treatment with astaxanthin on the elevated plus-maze

Fig.1 depicts the acute administration of diazepam and astaxanthin on the mice to show the performance on the elevated plus maze test. Post hoc analysis showed that the dose of astaxanthin 1, 3, 5mg/kg of astaxanthin and diazepam (1mg/kg) significantly increased the time spent in open arms ($p<0.01$). Fig.2 shows the number of entries in open arm is quietly increased at the dose of 3mg/kg and 5 mg/kg of astaxanthin.

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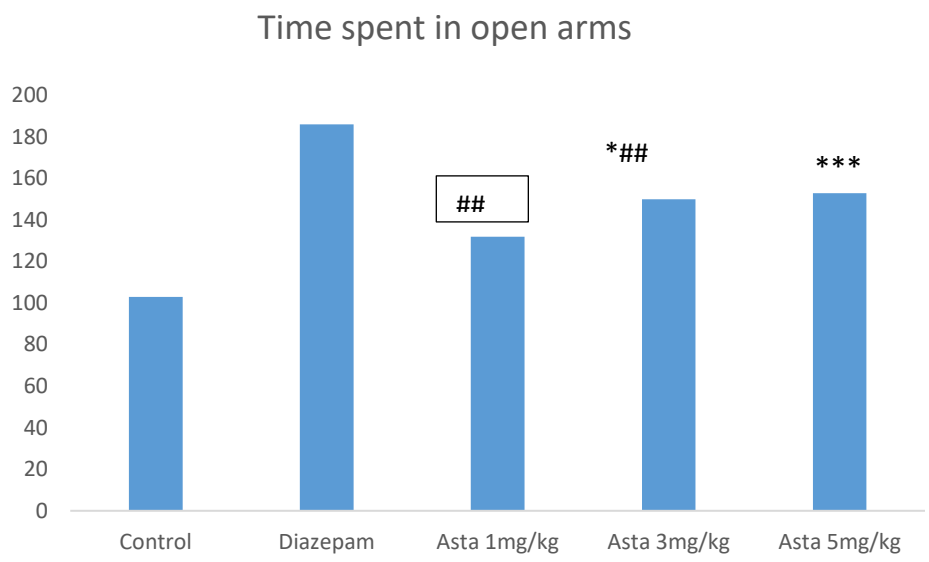

Figure 1: Effect of acute treatment with astaxanthin on the elevated plus-maze (time spent in open arms)

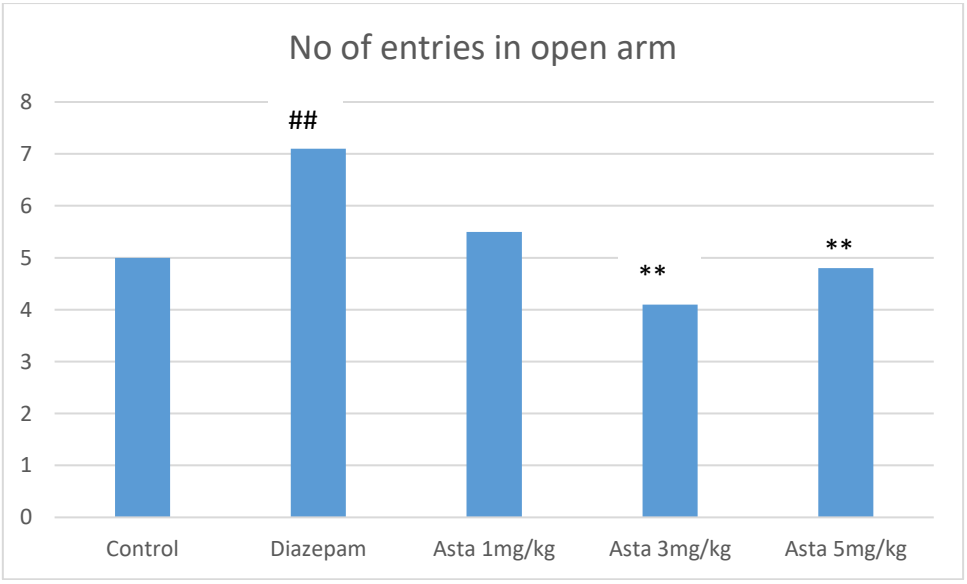

Figure 2

Fig.1 and 2 acute administration of astaxanthin and diazepam on elevated plus maze test.Astaxanthin (1, 3 and 5 mg/kg) and diazepam (1 mg/kg) were orally administered and performed on elevated plus maze test: time spent in open arms and no of entries are calculated each column represents mean±S.D. from six animals per group. Statistical analysis was performed by one-way ANOVA followed by Bonferroni multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001 (compared with diazepam group), #P<0.05, ##P<0.01, ###P<0.001 (compared with saline group).

The effect of acute treatment with astaxanthin on the hole board test

Fig.3 depicts the acute administration of diazepam and astaxanthin on the mice to show the performance on the hole board test. Post hoc analysis showed that the dose of astaxanthin 1mg/kg, 3mg/kg, 5mg/kg of astaxanthin and diazepam (1mg/kg) significantly increased the number of square crossings (p<0.01), Fig 4 represents the percentage of number of dipping's is increased with p value (p< 0.01) is considered significant as compared to a standard drug diazepam.

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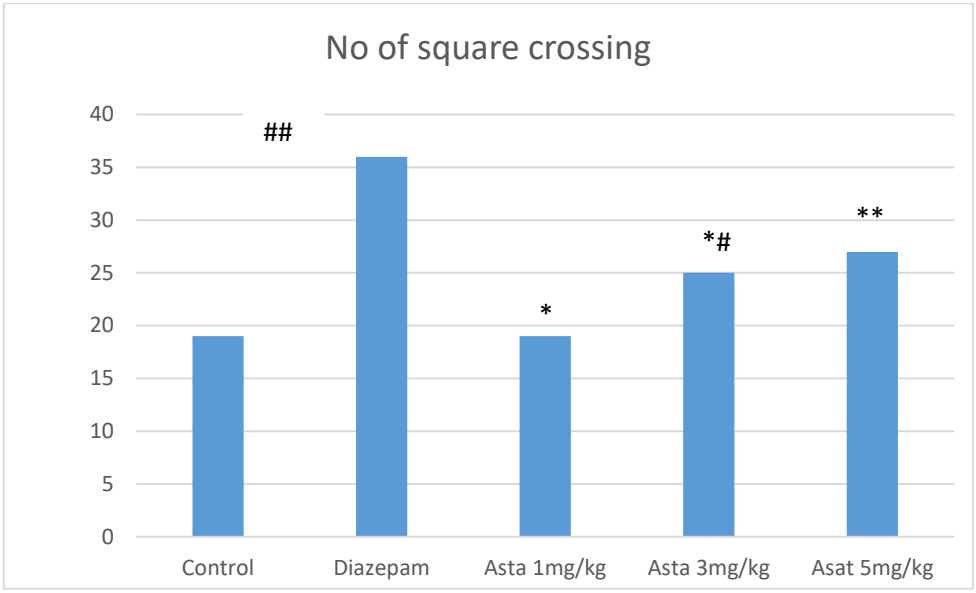

Figure 3: Effect of acute treatment with astaxanthin on the hole board test (No of square crossing)

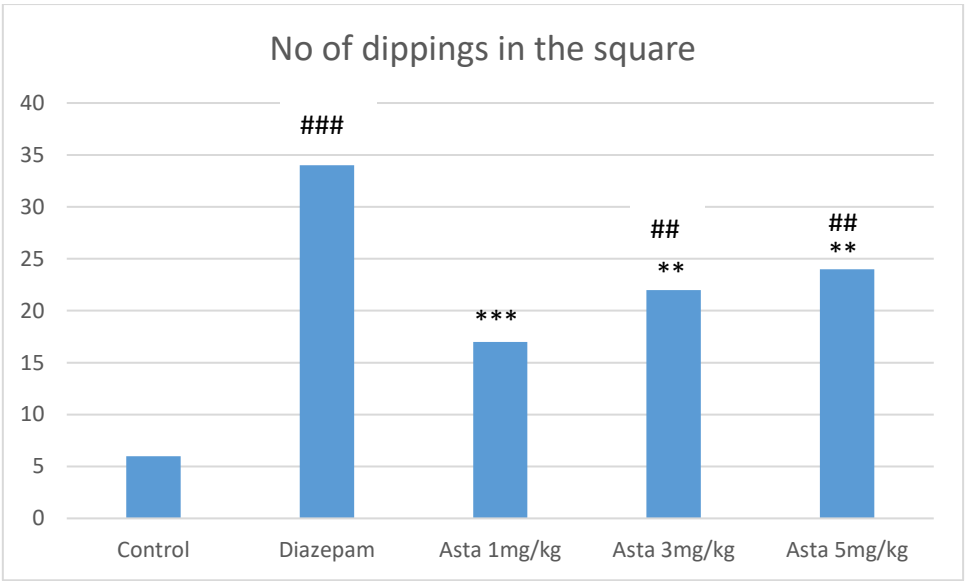

Figure 4

Fig.3 and 4 acute administration of astaxanthin and diazepam on elevated plus maze test.Astaxanthin (1, 3 and 5 mg/kg) and diazepam (1 mg/kg) were orally administered and performed on hole board test : no of dippings ,square crossed s are calculated. Each column represents mean±S.D. from six animals per group. Statistical analysis was performed by one-way ANOVA followed by Bonferroni multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001 (compared with diazepam group), #P<0.05, ##P<0.01, ###P<0.001 (compared with saline group).

The effect of chronic treatment with astaxanthin on the elevated plus-maze

Fig. 5 shows the effect of chronic administration (for 14 days) of astaxanthin at the doses of 1,3,5 mg/kg,p.o on the performance of mice on the elevated plus maze .Astaxanthin significantly increased the percentage of time spent in open arm on (p<o.o1) and entries in open arm (p< 0.01) (fig.6) as compared with diazepam .The dose 5 mg /kg showing increased performance which is taken for conducting the mechanism of action

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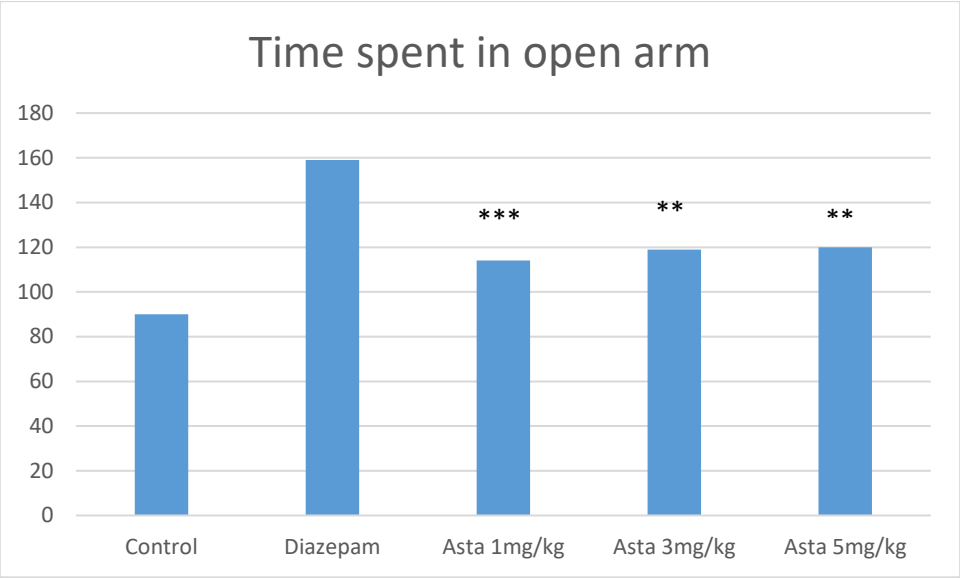

Figure 5: Effect of chronic treatment with astaxanthin on the elevated plus-maze (time spent in open arms)

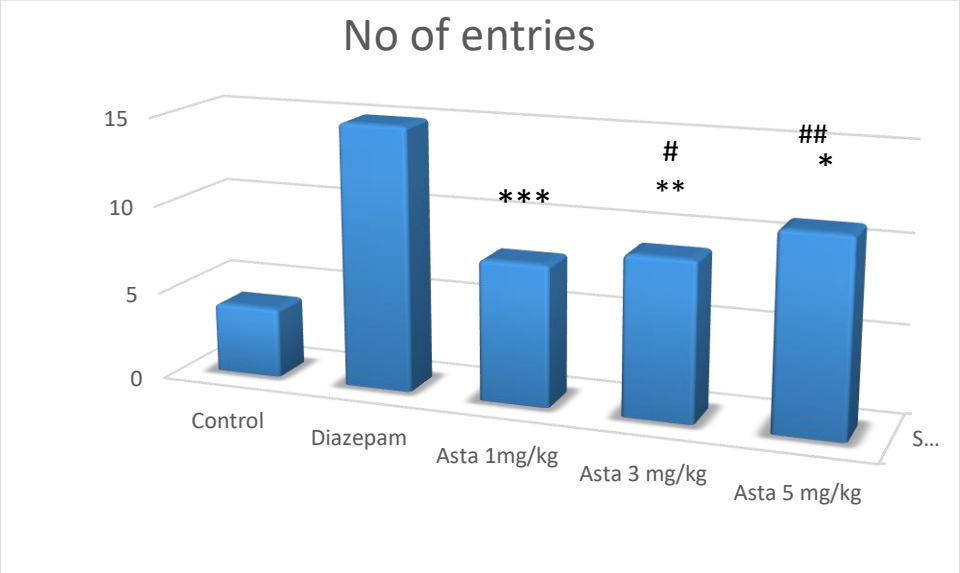

Figure 6

Fig.5 and 6 Effect of chronic administration of astaxanthin and diazepam on elevated plus maze test.Astaxanthin (1, 3 and 5 mg/kg) and diazepam (1 mg/kg) were orally administered daily for 14 days and on the 15th day on elevated plus maze test was conducted: time spent in open arms and no of entries are calculated Each column represents mean±S.D. from six animals per group. Statistical analysis was performed by one-way ANOVA followed by Bonferroni multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001 (compared with diazepam group), #P<0.05, ##P<0.01, ###P<0.001 (compared with saline group).

The effect of chronic treatment with astaxanthin on the hole board test

Fig. 7 and 8 shows the effect of chronic administration (for 14 days) of astaxanthin at the doses of 1, 3,5 mg/kg,p.o on the performance of mice on the hole board test .Astaxanthin significantly increased the percentage of square crossings on (p<o.o1) and dippings (p< 0.01) as compared with diazepam

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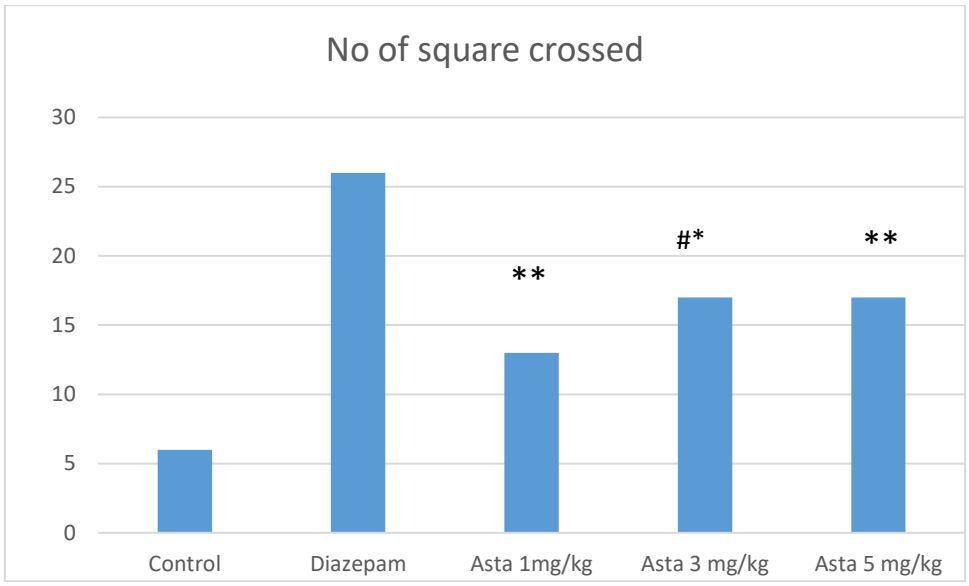

Figure 7: Shows the effect of chronic administration of astaxanthin on hole board test (no of square crossed)

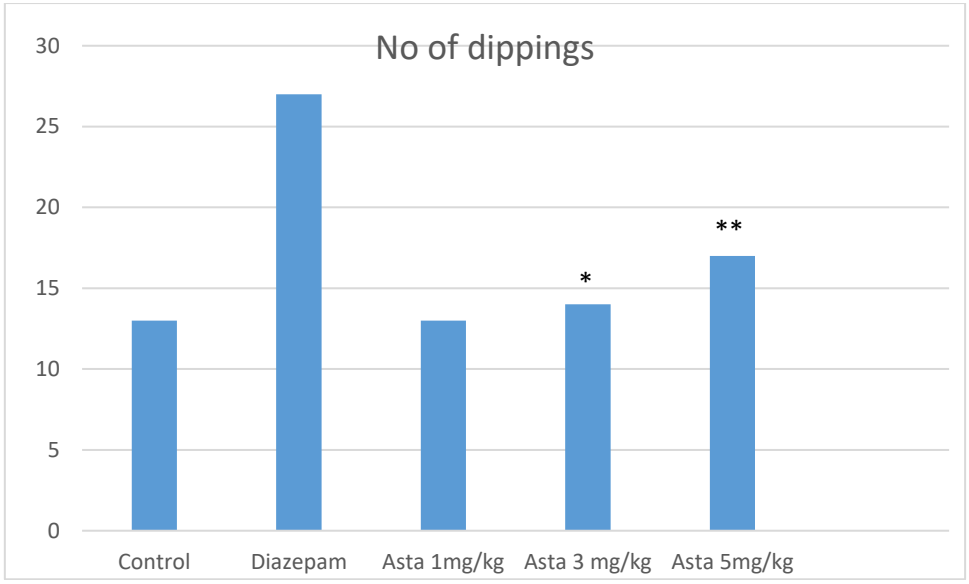

Figure 8

Fig.7 and 8 shows Effect of chronic administration of astaxanthin and diazepam on hole board test Astaxanthin (1,3 and 5 mg/kg) and diazepam (1 mg/kg) were orally administered daily for 14 days and on the 15th day hole board test was conducted: No of square crossed and head dippings are calculated Each column represents mean±S.D. from six animals per group. Statistical analysis was performed by one-way ANOVA followed by Bonferroni multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001 (compared with diazepam group), #P<0.05, ##P<0.01, ###P<0.001 (compared with saline group).

Effects of pre-treatment with GABA-acting drugs

Picrotoxin/flumazenil was administered 30 min before astaxanthin 5mg/kg and the behavioral parameters were evaluated using the elevated plus-maze (time spent in open arms) 30 min after astaxanthin administration. The results fig 9 and 10 shows effect of pre-treatment with Picrotoxins and flumazenil in mice was not able to block the antianxiety activity of astaxanthin with a dose 5mg/kg

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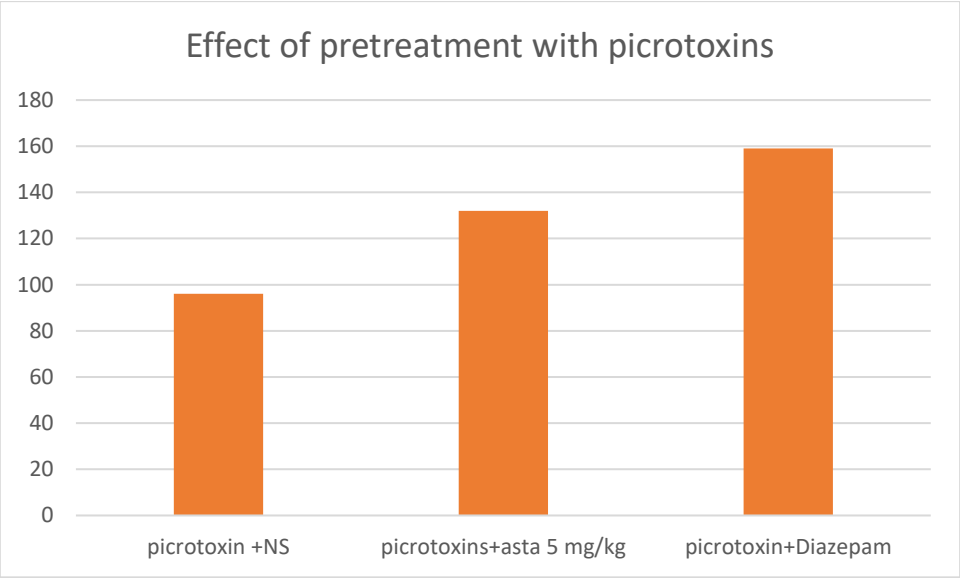

Figure 9

Fig 9: Effect of pre-treatment of mice with picrotoxin: time spent on open arms, no of entries into the open arms. Each column represents mean±S.D. from six animals per group. Statistical analysis was performed by two-way ANOVA followed by Bonferroni multiple comparisons test separately for astaxanthin picrotoxin interaction and diazepam picrotoxin interaction. No significant interaction is observed.

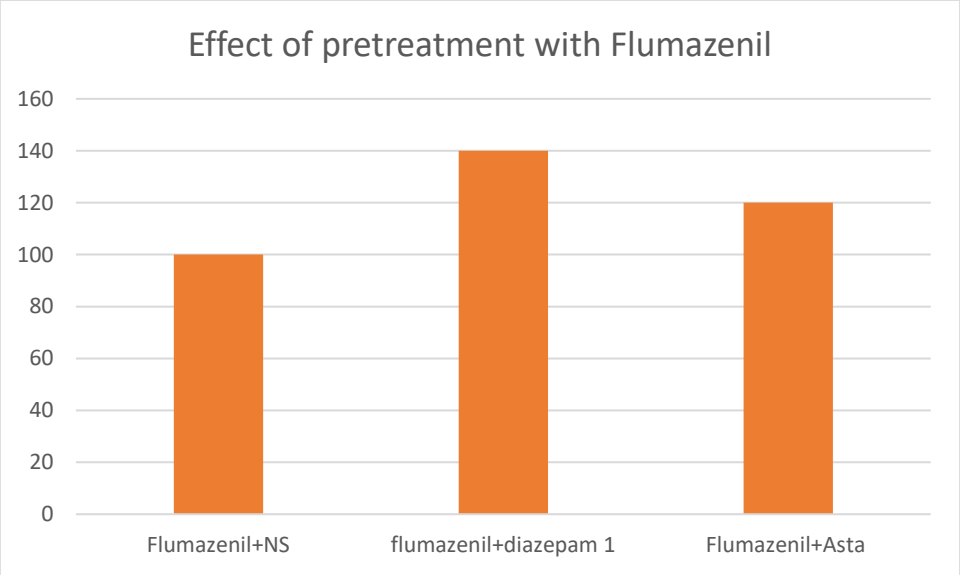

Figure 10

Fig. 10 Effect of pre-treatment of mice with Flumazenil: time spent on open arms, no of entries into the open arms Each column represents mean±S.D. from six animals per group. Statistical analysis was performed by two-way ANOVA followed by Bonferroni multiple comparisons test separately for astaxanthin flumazenil interaction and diazepam flumazenil interaction. No significant interaction is observed

Effect of pre-treatment with PCPA

A set of mice received an injection of PCPA (100 mg/kg i.e., a serotonin synthesis inhibitor) once daily for four successive days as pretreatment. 30 min after injection of PCPA, mice were treated with either a vehicle or Astaxanthin and performed on the elevated plus maze. Further, to investigate the other involvement of 5-

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HT1A/1B receptor in the antianxiety-like effect of Astaxanthin, animals were pretreated with pindolol (10 mg/kg, i.p., a 5-HT1A/1B receptor antagonist) After 30 min of pre-treated pindolol, they received astaxanthin or vehicle and performed on elevated plus-maze 30 min later. fig.11 and 12 depicts the increased percentage time spent on open arms by Astaxanthin treatment (5 mg/kg, p.o.) was significantly prevented by pretreatment of mice with PCPA (100mg/kg, i.p.) by using two-way anova ($p<0.01$).

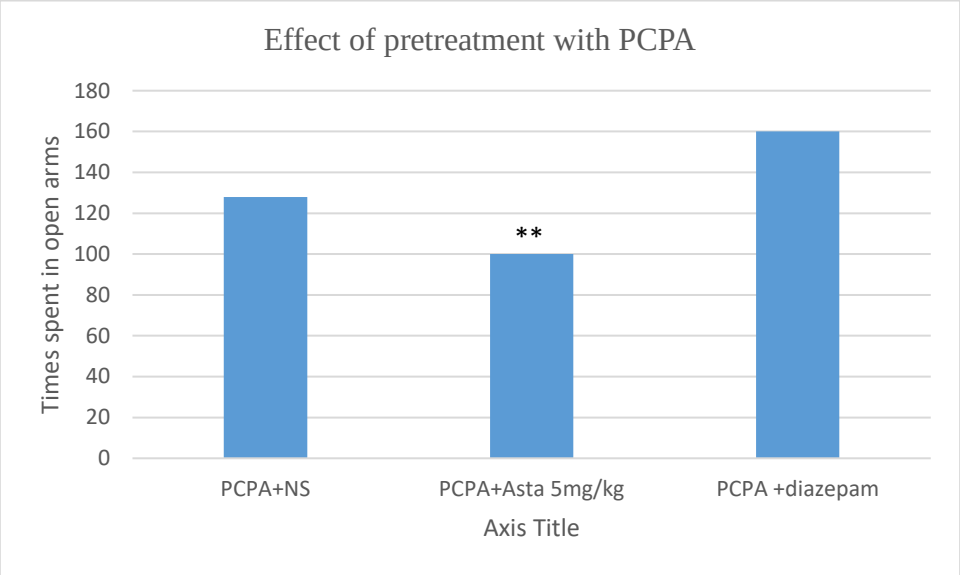

Figure 11

Fig 11. Effect of pre-treatment of mice with PCPA: time spent on open arms, no of entries into the open arms. Each column represents mean±S.D. from six animals per group. Statistical analysis was performed by two-way ANOVA followed by Bonferroni multiple comparisons test separately for astaxanthin picrotoxin interaction and diazepam picrotoxin interaction. *P<0.05, **P<0.01, ***P<0.001 (* denotes Astaxanthin interaction with Diazepam interaction)

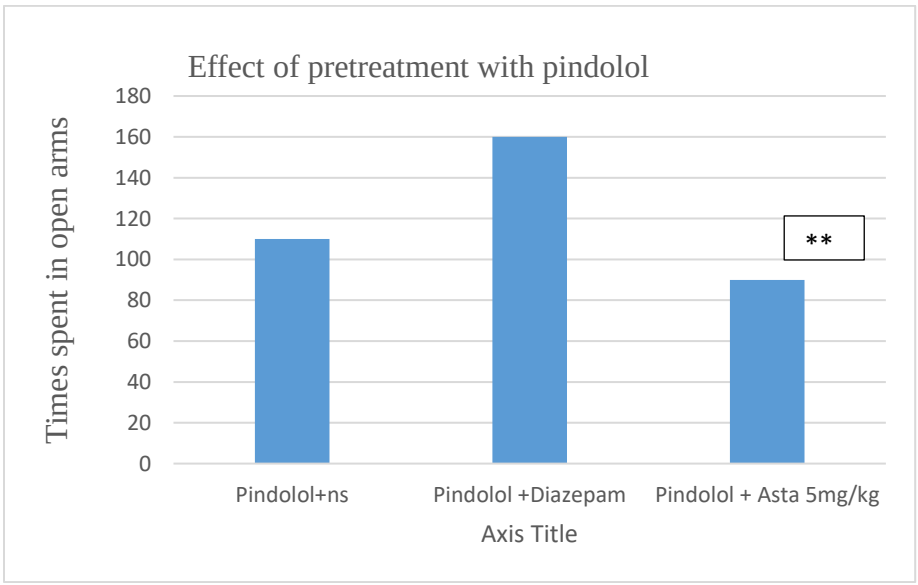

Figure 12

Fig 12 shows the Effect of pre-treatment of mice with pindolol: time spent on open arms, no of entries into the open arms. Each column represents mean±S.D. from six animals per group. Statistical analysis was performed by two-way ANOVA followed by Bonferroni multiple comparisons test separately for astaxanthin picrotoxin

**

interaction and diazepam picrotoxin interaction. *P<0.05, **P<0.01, ***P<0.001 (* denotes Astaxanthin pindolol interaction compared with Diazepam pindolol interaction group).

DISCUSSION

In this study, we examined the effect of astaxanthin over anxiety and its possible mechanism in mice models. Astaxanthin showed antianxiety-like effects on mice behaviour in the elevated plus maze test and hole-board test. Studies suggest that astaxanthin may play a beneficial role in oxidative stress-induced neural cell death in retina and brain.[8] astaxanthin studies has reported that it has a property to protect against ischemia/reperfusion-induced neurodegeneration in brain [9], have effect on cognition [10], improve mice memory. Previous report also stated that astaxanthin has blocking action on ethanol induced cortical depression, this indicates that astaxanthin may have effect on the neuronal and glial depolarization and neurovascular regulation in CNS[11]. Few studies have proved that astaxanthin has antianxiety property. Astaxanthin considered as ''pure antioxidant,'' unlike certain other carotenoids such as b-carotene, lutein, and lycopene that may show ''pro-oxidative'' characteristics under certain conditions.[12] Astaxanthin gained attention in experimental animal models due to its neuroprotective effect because of its strongest antioxidant property and lipid soluble keto carotenoid. [13] This is the first study to prove the possible mechanism of astaxanthin over antianxiety. In our study we have used 1, 3,5mg/kg of astaxanthin. Among these 5mg/kg is showing positive response over anxiety effect which is taken for finding the mechanism of action. Astaxanthin increases the time spent in open arms and number of entries in open arm in both acute and chronic study. It also significantly increases the dipping's and square crossed in hole board test. impairment in serotonin levels is one of the stongest reason for using SSRIs s therapeutic regimen.[14] Prolonged stress and anxiety stimulate the serotonergic structures such as frontal cortex, hypothalamus, amygdala and hippocampus .Due to continuous stimulation, it is believed to desensitize presynaptic 5-HT1a receptors and cause an indirect disruption in benzodiazepine GABA receptors, which may further potentiate anxiety[15]Buspirone, an antianxiety drug with partial agonist at 5-HT_{1A} receptor, used in the treatment of patients with generalized anxiety disorder.[16] A study done by Xi Jiang et.al stated that astaxanthin increases the 5-HT levels in serotonergic pathways. These effects were similar to that of the positive drugs such as imipramine and fluoxetine.[17] Furthermore it also affects the activation of indoleamine 2, 3-dioxygenase (IDO). IDO is the rate limiting step in tyramine metabolism. Activation of this enzyme is responsible for the pathophysiology of development of anxiety. Blockade of IDO inhibits the onset of lipopolysaccharide-induced depressive-like behavior in mice.[18] so, anxiety may be one of the reasons for development of depression. Therefore, the 5-HT_{1A} receptor could be a pharmacological target that is relevant to the mechanism of action of antianxiety drugs. In our study the anxiolytic activity of astaxanthin was blocked by PCPA. In order to confirm the role of 5-HT1A/1B receptor, the effect of pindolol (10 mg/kg, i.e., a β-adrenoceptor blocker/5- HT1A/1B receptor antagonist) is antagonizing the anti-anxiety like effect of Astaxanthin was carried out. The anxiolytic effect exerted by Astaxanthin was blocked by PCPA or pindolol, suggesting the serotonergic mediated action of this xanthophylls. The anxiolytic activity of astaxanthin was not blocked by GABA mediated drugs.

CONCLUSION

The present study indicates that astaxanthin produces anxiolytic effect with 5mg/kg on experimental models possibly via serotonergic pathway may be consider for future use in human

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