

## Neurobehavioral effect of alprazolam in presence of Ascorbic acid using albino rats

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### ABSTRACT

Alprazolam is used in the treatment of generalized anxiety, panic attacks with or without agoraphobia and depression. The physiological functions of Ascorbic acid are largely dependent on its oxido-reduction properties. Gamma aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian brain, and is the major neurotransmitters involved in the development of anxiety. The present study investigates the effects of Alprazolam and Ascorbic acid each alone and in combination on anxiety behaviour and on GABA levels in discrete brain regions of albino rats. Rats were divided into six animals groups (n=10): group 1, the control group, received only a single dose of 1% Tween 80; group 2, received a single dose of Alprazolam 2mg/kg; group 3, received a single dose of 125mg/kg of Ascorbic acid; group 4, received a single dose of 500mg/kg of Ascorbic acid; group 5, received a combination of Alprazolam and Ascorbic acid (125mg/kg); group 6, received a combination of Alprazolam and Ascorbic acid (500mg/kg). Scoring was performed immediately after 30 minutes of drugs administration. Elevated plus maze was used to evaluate the changes in behavior; brain GABA and Ascorbic acid levels were measured by HPLC. Results indicate that Ascorbic acid produced dose dependent anxiolytic effects; it has no sedative effect up to the dose 500mg/kg. Alprazolam produced anxiolytic action without sedation. The combined treatment of Ascorbic acid with Alprazolam did not potentiate the anxiolytic action, but it has additive effect. Acute administration of Ascorbic acid was accompanied by an increase in GABA levels in almost all brain areas studied, leading to anxiolytic action. Ascorbic acid may act as a partial allosteric modulator of the GABAA receptor, since it induces a smaller response in their target cells than do full allosteric modulator Alprazolam. Acute administration of Ascorbic acid (125 or 500mg/kg), selectively increases the Ascorbate levels in striatum, mid-brain and cerebral cortex. Alprazolam did not change Ascorbate levels in all brain areas studied.

**Key words:** Ascorbic acid, Alprazolam, GABA EPM, Behavior.

### INTRODUCTION

Anxiety and depression are common, stress-induced, psychiatric disorders [1]. Anxiety disorders and mood disorders are associated with pathogenic mechanisms involved with the oxidative pathway [2]. A link was found between oxidative stress and Obsessive-Compulsive Disorder and Panic Disorder, indicating that oxidative metabolism can affect the regulation of anxiety[3].

Since its introduction in 1960, Alprazolam became one of the most used and most often prescribed psychoactive drug. Alprazolam (8-chloro-1-methyl-6-phenyl-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine) is a potent short acting benzodiazepine (BZ) used for the treatment of anxiety disorders [4]. Alprazolam binds non-selectively to GABAA-benzodiazepine receptor complex. At the receptor complex, Alprazolam facilitates the binding of Gamma aminobutyric acid (GABA) and increase the influx of chloride ions. The presence of GABAA, in turn,

inhibits the action of several connected brain structures. The inhibition exerted by GABA results in a general slowing of brain activity. Furthermore, GABA system interacts with other neurotransmitter systems, including noradrenergic, serotonergic, cholinergic, and opioidergic systems. Especially Alprazolam's interaction with the serotonergic and noradrenergic pathways to the limbic system and brain stem structures (e.g., locus ceruleus) contribute to its clinical effectiveness in the treatment of anxiety and depression [5].

GABA is the major inhibitory neurotransmitter in the mammalian brain [6], occurring in 30-40% of all synapses. Concentration of GABA in the brain is 200-1000 times greater than that of the monoamines or acetylcholine [7]. It has been revealed that GABA is the major neurotransmitters involved in the development of anxiety. In consistency with this finding, it was found that the disturbance of Corticotropin-releasing factor (CRF) containing GABA(A) $\alpha$ 1 neurons causes increase in anxiety and impaired fear extinction, both of which are

symptoms diagnostic for anxiety disorder, such as posttraumatic stress disorder [8]. The locus ceruleus and raphe nuclei are areas of the brain likely to be involved in anxiety disorders. The limbic system, which receives information from these areas of the brain, has many benzodiazepine receptor-binding sites. Fadem [9] found that, the temporal and frontal cerebral cortex is associated with anxiety behavior.

Ascorbic acid (vitamin C) is an antioxidant involved in anxiety, memory, fatigue and mood state studies. Some animals and humans, cannot synthesize ascorbic acid due to the lacking of, the L-glulonolactone oxidase enzyme [10].

Vitamin C may be useful in preventing the diazepam-induced immunosuppression [11]. It provides protection to young animals from diazepam-induced impairment of memory. Vitamin C has shown promise as a powerful memory-improving agent particularly effective in aged animals. The underlying mechanism of action of Vitamin C may be attributed to its antioxidant property [12].

Vitamin C supplement increase of its plasma levels and reduce anxiety. It was proved that Vitamin C plays an important therapeutic role for anxiety [13]. Aburawi [14] found that Ascorbic acid with antidepressants significantly decreased the total Hamilton depression rating scale; therapeutically, Vitamin C was beneficial with antidepressants in the treatment of depression, and showed a good response in treated patients using combined Vitamin C with antidepressants. In addition, it has been demonstrated that Ascorbic acid improved the efficiency of fluoxetine to treat depression.

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Ascorbic acid is a redox agent, it is highly concentrated in the retina and other regions of the CNS, and accumulated in neurons and glial cells by specific transporters. In the retina, Ascorbic acid levels rise above 100 times the concentration found in blood plasma [15]. Moreover, the brain contains the highest level of Ascorbate in the body and there are active uptake mechanisms in the choroid plexus and cell membrane to maintain intracellular levels at 16-25 times higher than blood levels [16].

Interestingly, the extracellular concentration of Ascorbic acid can transiently undergo substantial increases during neuronal activity. Levels of extracellular brain Ascorbate vary greatly according to the activity of the animal, being lowest during sleep and highest with prolonged activity and stress. In retinal neurons, the ascorbate transporter SVCT2 (sodium-dependent vitamin C transporter 2) mediates extensive sodium-dependent Ascorbic acid extrusion through a mechanism regulated by neuronal

depolarization and glutamate concentrations. Furthermore, Ascorbic acid has been shown to modulate the activity of NMDA glutamate receptors and voltage-activated ion channels [15].

The aim of this study is to investigate the Neurobehavioral effect of alprazolam in the presence of Ascorbic acid using albino rats.

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## Materials and Methods

### Materials

#### Drugs and chemicals

Ascorbic acid was supplied by Weisheng Pharmaceutical Company, Shrijiazhuang, Korea; Alprazolam was obtained through The Upjohn Company, Cairo, Egypt; Acetonitrile and Methanol (HPLC grade) was bought from SDS, France; Phosphoric acid, Tween 80 were obtained from Riedel-De Haen AG, Seelze Hanover, Germany. Perchloric acid was purchased from May & Baker Ltd, England; Potassium carbonate and Sodium hydrogen carbonate from BDH Limited, England. Dansyle chloride (5-dimethylamino naphthalene-1-sulfonyl chloride) and  $\gamma$ -Amino-n-Valeric acid (Valine; 5 Aminopentanoic acid),  $\gamma$ -Amino-n-Butyric acid (GABA; 4-Aminobutanoic acid; Piperidic acid) were purchased from Sigma Chemical Company, USA; Theobromine (3:7-dimethyl-xanthine) and anhydrous acetone from Koch-Light Ltd, England.

### Animals

Male Albino Wistar rats weighing between 250 to 320 grams, were bred in the animal house of the Faculty of Pharmacy, University of Tripoli, Tripoli - Libya. They were housed under controlled conditions (20-25°C) and 12 hours light/ dark cycle. Animals received a standard pellet diet (Beeky company-Austria) and water ad libitum.

### Drugs administration

Animals were randomly assigned to receive different treatments or vehicle. Acute drug administration was performed by intraperitoneal route. Tween 80 (1%) was used as suspending agent [17], and all control animals were injected with the corresponding vehicle. A volume of injection of 1 ml/kg of body weight was adopted for all experiments [18].

## METHODS

### Alprazolam action in presence of Ascorbic acid

Rats were divided into six equal groups of 10 animals each and treated as following: Group I, the control group received a single dose of 1% Tween 80; Group II, received a single dose of 2mg/kg Alprazolam; Group III, treated with a single dose of 125mg/kg Ascorbic acid; Group IV, received a single dose of 500mg/kg Ascorbic acid; Group V, treated with a combination of 2mg/kg

Alprazolam and 125mg/kg Ascorbic acid; Group VI, received a combination of 2mg/kg Alprazolam and 500mg/kg Ascorbic acid. Behavioral measurements using plus maze were scored 30 minutes after drug administration. The brain homogenate were prepared for GABA and Ascorbic acid levels measurements immediately after behavioral test.

### Behavioural measurements

Wooden elevated plus-maze (EPM) apparatus (Fig. 1) consisted of two open arms (45×10 cm each) and two opposite closed arms of the same size, with walls of 40 cm height. The arms linked by a central 10×10 cm square. The maze was suspended 50 cm from the room floor [19, 20]. Plus-maze test was conducted in a closed room with low illumination [21]. The test was performed in the same room between 8:00am-16:00pm, under constant conditions, including temperature, humidity and the natural light/dark cycle. Animals were placed on the central part of the maze facing closed arm. The number of entries, lines crossed and the time spent in the open and closed arms were scored during 4 minutes. An entry defined as having both forepaws in the respective arm. The line crossing defined as both forepaws crossing the line [22, 23]. Anxiety measure (A.M) was calculated by dividing the total time spent in closed arms by total time of the test; when the animal suffers fear and anxiety, A.M ratio is closed to 1 [24].

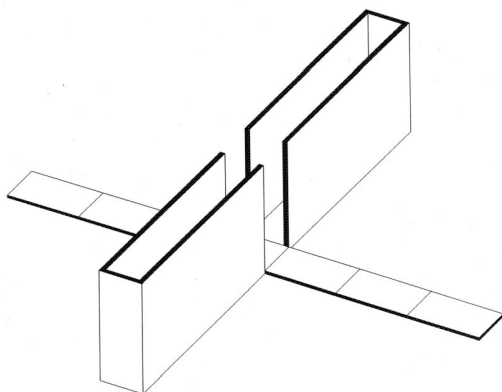


Figure 1: Plus Maze

### Brain dissection, homogenization and preservation

#### Dissection

The method of dissection was previously described by Elhwuegi (1981) [25] and Aburawi et al., (2000) [26]. Rats were sacrificed by cervical dislocation and decapitation. The body was exposed to microwave irradiation for 4 seconds [27 - 29]. The brain were quickly removed on glass plate resting over crushed ice. Cerebellum was removed first by cutting the cerebellar peduncles, followed by the pons and medulla (brain stem)

which were separated from the rest of the brain. Cerebral cortex was then pulled away to expose the corpus callosum and the lateral ventricles. Striata protruding from the inner surface of the cerebral hemispheres into the ventricles together with the caudate nuclei, were removed by gently scraping the inside of the ventricle with a sharp scalpel. Cerebral hemispheres were then dissected from the rest of the brain. The rest of the brain referred to as "mid brain" includes the midbrain proper, the thalamus, hypothalamus and hippocampus.

#### Homogenization and preservation

The removed brain regions were placed after weighing in 100 ml plastic tubes previously placed in iced bath and containing 10 ml of ice-cooled 0.1 M Perchloric acid and 1ml of 150µg/ml valine as internal standard. The tissues were homogenized (using the homogenizer Ultra-Turrax Janke and Kunkel AG: Germany) for one minute, keeping the tube embedded in an ice bath, and then centrifuged at 5000 rpm for 10 minutes at 4 C°. The supernatants were stored at -20C° until assayed [27 - 29].

#### Measurement of GABA levels

##### Calibration curves

Calibration curves were constructed by carrying standard solutions of GABA (0, 50, 100, 200 and 300 µg/ml) in 0.1 M Perchloric acid, each contains 150µg/ml of valine as an internal standard. One millilitre of each standard solution was diluted to 10 ml with 0.1 M Perchloric acid.

##### Dansylation reaction

Dansylation reaction was induced using the method of [29]. Dansylation was carried out by taking 100 µl of the supernatant of the samples or the standards and added to a micro-tube containing 100 µl of 0.1M potassium carbonate solution. These solutions were mixed using vortex mixer and then centrifuged using micro centrifuge at 10,000 rpm for 10 minutes. The supernatant (100 µl) of the centrifuged mixture was transferred into a Pyrex tube containing 100 µl of 0.1 M sodium hydrogen carbonate solution, and then 400 µl of working dansyle chloride solution were added. The tubes were shaken for 30 seconds using vortex mixer and then incubated at 90C° in bench top oven for 30 minutes. The tubes were not capped during the incubations, to allow much of the sample to evaporate during incubation. This did not appear to adversely affect the progress of the dansylation reaction and served to concentrate the samples. After getting the tubes out of the oven, they were left to cool down to room temperature, and the dansylated derivatives were transferred into 1.5 ml micro tubes and stored at -20C° until assayed.

##### Liquid chromatography

High performance liquid chromatography system was used to resolve and quantify the samples [28], consisted of Cecil binary gradient system, pump [CE 1100 pump, made by Cecil Instruments Limited, England], connected

to PC with data control software to acquire and analyze chromatography data. The HPLC column was 5  $\mu$ m, 250 $\times$ 3.2 mm C18 ODS, from Hi-chrome Waters, England. The fluorometric detector was made by Phillips, Japan.

Mobile phase consisted of water-acetonitrile (HPLC grade) mixture (65:35 v/v) containing 0.15 % (v/v) phosphoric acid, filtered using 0.45 $\mu$ m pore size membrane filter and degassed by helium. The flow rate was 1.0 ml/min. The detector excitation was kept at 333nm and emission at 532nm [29].

25 $\mu$ l of the dansyle derivative of GABA sample was transferred to HPLC micro sample vials and injected into the column. The peak ratios of the samples were calculated with reference to the internal standard. The concentration of the samples was calculated from concentration-peak ratio curve of dansylated standards. Linear regression and samples concentration were calculated using windows (Excel) software package. Retention time of GABA and internal standard were in the range of 6.43 and 7.51 minutes.

#### Measurement of Ascorbic acid in the brain.

##### Calibration curves

Calibration curves were constructed by carrying solutions of standard Ascorbic acid (0, 50, 100, 200 and 400  $\mu$ g/ml) in 0.1 M Perchloric acid, each containing 100  $\mu$ g/ml of Theobromine as internal standard. One millilitre of each standard solution diluted to 10 ml of Perchloric acid.

Liquid chromatography.

Measurement of Ascorbic acid was performed using modified method of polymer laboratories, 2003. The HPLC system was used to resolve and quantify the samples consisted of reversed phase column (5  $\mu$ m, 250 $\times$ 3.2 mm) C18 ODS from Hi-chrome Waters, England. The spectrophotometer detector made by Cecil 1200, England. The HPLC mobile phase composed of 0.1M Perchloric acid – acetonitrile mixture (89:11 v/v). The flow rate was 1.0 ml/min. The spectrophotometer wavelength was 270 nm.

The standard solution (25  $\mu$ l) or brain homogenate supernatants was transferred to HPLC micro-sample vials and injected into the column. The peak ratios of the samples were calculated with reference to the internal standard. The concentration of the samples was calculated from the concentration peak ratio curves of standards. The peak ratio of the standards were linear from 50 to 400  $\mu$ g/ml for Ascorbic acid ( $R=0.93$ ). Linear regression and samples concentration calculated using an Excel software package. Retention time of Ascorbic acid and internal standard were in the range of 2.33 and 5.40 minutes.

Statistical analysis.

Descriptive statistical analysis was applied on parameters of different samples using SPSS-12. Kolmogorov-Smirnov maximum deviation test, for goodness of fit to find out whether the observed samples were parametric. If

the parameters normally distributed, the difference between groups analyzed using one-way ANOVA, Followed by post hoc test (Duncan and LSD tests). If the parameters of samples were non parametric, treatments were compared by applying Mann-Whitney two samples (none matched) test. The difference was considered to be significant at  $p \leq 0.05$ .

## RESULTS

### Alprazolam behavioral effect in presence of Ascorbic acid using plus maze

Table 1 shows the effects of acute administration of Alprazolam (2mg/kg) or Ascorbic acid (125, 500mg/kg) on rat behaviour, using EPM test. All treatment produce a significant decrease in anxiety measure compared to the control treated group. Ascorbic acid (500mg/kg) alone significantly decrease anxiety measure compared to Ascorbic acid (125mg/kg) alone. The combined treatment of Alprazolam with Ascorbic acid (125mg/kg) significantly decreased the anxiety measure compared to Alprazolam alone or Ascorbic acid (125mg/kg) alone. The combined treatment of Alprazolam with Ascorbic acid (500mg/kg) produce a decrease in anxiety measure compared to Alprazolam treated group.

Treatment with Alprazolam alone or Ascorbic acid (125mg/kg) alone significantly increase the total lines crossed in all arms compared to the control group; however, the combined treatments of Alprazolam with Ascorbic acid 125mg/kg decreased the total lines crossed significantly compared to Alprazolam alone or to Ascorbic acid (125mg/kg) alone treated groups. Ascorbic acid administration in a dose of 500mg/kg combined with Alprazolam significantly decreased the total lines crossed compared to Alprazolam treated group.

The number of entries into the open arms increased significantly by all the treatments compared to the control group; while the administration of Ascorbic acid (500mg/kg) alone or Ascorbic acid (125mg/kg) combined with Alprazolam decreased the number of entries into open arms compared to Ascorbic acid (125mg/kg) alone treated group. In the closed arms, Ascorbic acid (125 mg/kg) alone significantly increase the number of entries, while the combination of Alprazolam with Ascorbic acid (125 or 500mg/kg) treated groups significantly decrease the number of entries into the closed arms compared to the control group. The combined treatments of Alprazolam with Ascorbic acid (125mg/kg) significantly decrease the number of entries into the closed arms compared to Ascorbic acid (125mg/kg) alone treated groups; while the combined treatment of Alprazolam and Ascorbic acid (500mg/kg) significantly decrease the number of entries into the closed arms compared to Alprazolam alone treated group. Alprazolam alone or Ascorbic acid (125mg/kg) alone significantly increased the total number of entries into the open and closed arms compared to the control group. The combined treatment

of Alprazolam with Ascorbic acid (125mg/kg) decreased the total number of entries significantly compared to Ascorbic acid (125mg/kg) alone treated groups. The combined treatment of Alprazolam and with Ascorbic acid (500mg/kg) significantly decreased the total number of entries compared to Alprazolam treated group.

**Effect of Alprazolam and Ascorbic acid on rat brain GABA levels**

The effect of acute administration of Alprazolam or Ascorbic acid each alone or when combined together on GABA brain levels was presented in table 2. The analysis of the data of different groups by LSD multiple comparison test resulted in an over all significant increase in GABA levels in cerebellum, brain stem, mid brain, cerebral cortex and whole brain when compared to the control group.

In striatum, the combination of Alprazolam with Ascorbic acid (125 and 500 mg/kg) significantly decreased GABA levels compared to Alprazolam alone treated group, while in cerebral cortex the dose 500 mg/kg Ascorbic acid did not produce any change in GABA levels compared to Alprazolam alone treated group.

**Effect of acute administration of Alprazolam and Ascorbic acid on Ascorbic acid levels in different brain regions**

Results and the statistical analysis for the effect of Alprazolam and Ascorbic acid on Ascorbic acid levels in different rat brain areas are shown in table 3. Acute administration of Alprazolam (2mg/kg) alone, or Ascorbic acid (125, 500 mg/kg) alone or when combined with Alprazolam did not change the Ascorbate levels in

Table: 1  
Effect of acute administration of Alprazolam and Ascorbic acid on behavior using Plus Maze

Treatment	Anxiety measure	Lines crossed in open and closed arms	Total entries into open and closed arms
T80 1% (1ml / kg)	1.0±0.0	7.0±1.51	1.2±0.2
ALPRAZOLAM (2mg/kg)	0.437±0.0975 *	17.7±1.61 *	3.2±0.51 *
ASCORBIC ACID (125mg/kg)	0.421±0.0784 *	19.2±3.63 *	4.3±0.78 *
ASCORBIC ACID (500mg/kg)	0.174±0.0737 *,b	14.0±3.29	2.4±0.48 b
ALP+ASC (125mg/kg)	0.143±0.075 *, a, b	9.8±2.02 a,b	2.0±0.52 b
ALP+ASC (500mg/kg)	0.0475±0.0324*,a	7.2±1.95 a	1.6±0.27 a

The values are the means ± S.E. for 10 rats; (\*) significantly different from T80 treated group at p≤0.05; (a) Significantly different from Alprazolam treated group at p≤0.05; (b) Significantly different from Ascorbic acid 125mg/kg treated group at p≤0.05.

Table: 2  
Effect of Alprazolam and Ascorbic acid on rat brain GABA levels in different brain regions

TREATMENTS	CEREBELLUM	BRAIN STEM	STRIATUM	MID BRAIN	CEREBRAL CORTEX	WHOLE BRAIN
T80 1% 1ml/kg	16.2±2.5	14.8±2.2	22.0±2.9	15.0±2.0	17.8±1.0	17.1±0.9
Alprazolam 2mg/kg	27.5±1.7*	23.8±2.1*	87.4±6.0 *	44.0±8.3 *	34.4±5.7*	34.6±4.0*
Ascorbic acid (125mg/kg)	24.0±1.8*	22.6±2.5*	41.2±7.6 *	54.5±7.9 *	34.5±4.4 *	35.0±3.6*
Ascorbic acid (500mg/kg)	24.9±3.9*	22.8±3.3*	28.7±6.5	49.2±7.9 *	31.7±4.5	31.5±4.1 *
Alp + AsA (125mg/kg)	24.3±2.2*	23.4±1.8*	56.7±7.9 *,a	47.6±6.9 *	33.8±7.1 *	33.3±5.1 *
Alp+AsA (500mg/kg)	25.8±1.6*	22.7±1.5*	44.9±5.1 *,a	46.2±6.9 *	35.4±5.2 *	34.4±3.4*

The values are the means (µg/gm tissue weight) ± S.E. of GABA for 10 rats; (\*) significantly different from T80 treated group at p≤0.05; (a) significantly different from Alprazolam treated group at p≤0.05.

In striatum and cerebral cortex, Alprazolam alone treated group produced a highly significant increase in GABA levels compared to the control group. Ascorbic acid (125mg/kg) alone significantly increase GABA levels in striatum; while the combined treatment of Alprazolam with Ascorbic acid (125 or 500mg/kg) produced a significance increase in GABA levels compared to the control group. Ascorbic acid (500mg/kg) alone did not change GABA levels compared to the control treated

cerebellum and brain stem.

In striatum, the levels of Ascorbate were significantly increased after acute administration of Ascorbic acid (500 mg/kg) compared to the control group. The combined treatment of alprazolam with Ascorbic acid (125 or 500 mg/kg) significantly increased the levels of Ascorbate in striatum compared to control group and alprazolam alone treated group. This increase of Ascorbic acid was dose dependent.

In midbrain, administration of Ascorbic acid (500 mg/kg) alone and in combination with Alprazolam produced an increase in the levels of Ascorbate significantly when compared to the control or to Alprazolam alone treated groups.

food/water deprivation or electric shock [36, 39]. Despite or possibly by virtue of these advantages, there are probably as many EPM paradigms as there are laboratories using the model in preclinical anxiety research [35, 36]. This model is based on the natural

Table: 3  
Effect of acute administration of Alprazolam and Ascorbic acid on Ascorbic acid levels in different brain regions

TREATMENTS	CEREBELLUM	BRAIN STEM	STRIATUM	MID BRAIN	CEREBRAL CORTEX	WHOLE BRAIN
T80 1% 1ml/kg	23.0±2.4	22.3±4.0	43.8±17.6	20.2±2.6	36.1±1.8	29.0±1.2
Alprazolam (2mg/kg)	18.2±3.0	18.4±2.3	55.0±13.0	20.4±2.8	35.9±2.6	28.1±1.4
Ascorbic acid (125mg/kg)	44.2±15.6	25.3±6.8	133.5±62.0	39.3±6.3	44.3±2.0*,a	43.1±3.8*,a
Ascorbic acid (500mg/kg)	48.6±10.8	26.5±3.5	228.7±67.7*	52.9±6.5*,a	46.1±1.4*,a	49.0±3.4*,a
Alp + AsA (125mg/kg)	43.8±6.7	25.4±2.1	242.7±97.1*,a	38.3±9.9	45.0±2.7*,a	45.0±2.6*,a
Alp + AsA (500mg/kg)	48.1±17.8	28.8±6.4	296.5±89.6*,a	54.1±16.3*,a	46.3±2.2*,a	51.2±4.1*,a

The values are the means (µg/gm tissue weight) ± S.E. of Ascorbic acid level for 10 rats; (\*) significantly different from T80 treated group at p≤0.05; (a) Significantly different from Alprazolam treated group at p≤0.05.

The acute administration of Ascorbic acid (125, 500 mg/kg) each alone or in combination with Alprazolam (with low or high dose of Ascorbic acid) increased the levels of Ascorbate significantly when compared to control or to Alprazolam alone treated groups in both of cerebral cortex and the whole brain.

## DISCUSSION

The maze was suspended 50 cm from room floor, causing aversive stimuli to the animals in the open arms. Rats should be exposed to the plus maze only once after treatment, otherwise the anxiolytic-like effects are considerably reduced by pre-exposure to the maze [19, 20], this phenomenon is known as “one trial tolerance”. It was found that pre-exposure alters behavioral and pharmacological responses in the EPM [22, 30 - 32].

Tween 80 (Polysorbate 80), a nonionic surfactant and emulsifier, is used in this study to produce homogeneous dosage form to work with. The dansylation process is carried out by inserting a dansyl group in the GABA molecule, then measuring the fluorescence emitted from dansylated GABA using fluorometric detector.

### Effect of acute administration of Alprazolam and Ascorbic acid on behavior

Elevated Plus-Maze test (EPM) is the most popular of all currently available animal models to study experimental anxiety [33 - 36], and to screen new anxiolytic drugs [19, 35, 37, 38]. It is likely that the popularity of the EPM test, is due to its obvious and numerous advantages, namely: economy, rapidity, simplicity of design and bidirectional drug sensitivity, coupled with the fact that it does not require lengthy training procedures or the use of

aversion of rodents to open spaces [40, 41], and has been extensively validated using pharmacological and behavioral criteria [39, 42]. During a typical plus-maze test, animals will spend most of the time in the closed arms of the maze, a behavior, which seems to reflect their fear of open spaces [43].

Using plus maze model, acute administration of Alprazolam (2mg/kg) shows significant anxiolytic effect (Table 1), this may be due to the fact that the anxiolytic effect of benzodiazepines result from the ability of these agents to potentiate the action of GABA at the GABAA receptors [44]. Benzodiazepines induce conformational (allosteric) changes in the GABA binding site, thereby increasing the affinity of the receptor for GABA [45]. As a result, the frequency of chloride channel opening is increased resulting from the binding of GABA and the cell is further hyperpolarized, yielding a more pronounced decrease in cellular excitability [46]. Alprazolam in the dose used has no sedative effect; it did not change (i.e. decrease) the total lines crossed and the total number of entries into open and closed arms. The increase in the total lines crossed and the total number of entries due to alprazolam administration may be due to the increase in exploratory behavior.

Acute administration of Ascorbic acid in a dose of 125 or 500mg/kg produced an anxiolytic effect; this effect was demonstrated by a decrease in the anxiety measure (decrease in the time spent in closed arms, and increase in the time spent in open arms). The primary indices of anti anxiety are the frequency of entries and time spent by the animal in the open arms [34]. It was mentioned that the time spent and number of entries into open arms are negatively related to anxiety, while the time spent in the

closed arm is positively related [47]. Based on this, it is concluded that, the present results suggest that Ascorbic acid produced dose dependent anxiolytic-like effect using elevated plus maze (increase time spent in open arms; decrease time spent in closed arms, while the lines and number of entries into open arms was increased) without any sedative effect (total lines crossed and total entries was not decreased).

Neurochemical investigations have linked anxiety to the dysfunction in central GABAergic, serotonergic and noradrenergic systems [48]. Ascorbic acid has been shown to reduce behavioral signs of anxiety [49, 50], by modulate glutamatergic, dopaminergic [51 - 56], and noradrenergic activity [57, 58]. In addition to its functions as antioxidant in the CNS, Ascorbate has been shown to be a neuromodulator of both dopamine and glutamate-mediated neurotransmission [59, 60]. Ascorbate and glutamate have been shown to regulate behavior, thus, systemic injections of Ascorbate enhance or suppress the motor activity depending on the dose, since injection of high systemic [61], or intrastriatal [62] dose has a dopamine antagonist effect on behavior, opposing the action of amphetamine and enhancing that of haloperidol. However, Ascorbate, at relatively low doses, was reported to potentiate amphetamine-induced behavioral activation [63]. Ascorbate influences neostriatal glutamate transmission presynaptically via an Ascorbate-glutamate hetero-exchange at the glutamate reuptake site and perhaps postsynaptically via NMDA receptor blockade [64]. Because of its role in neuronal function and its high concentration in the brain especially striatum, Ascorbic acid may play a role in behavioral activation [65].

The anxiolytic effects of Ascorbic acid may be based on the fact that L-Ascorbic acid, increase neurosteroids synthesis and accumulation in the various rat brain regions, and this action of Ascorbic acid inhibited by 5-HT antagonists [66]. Neurosteroids are synthesized, independently from pituitary control, from cholesterol or by metabolism of circulating precursors [67]. Mitochondria of glial cells express the so-called "peripheral" or "mitochondrial" benzodiazepine receptor that shows high affinity for a subclass of benzodiazepines [68, 69]. The binding of benzodiazepines to the mitochondrial benzodiazepine receptor increases the access of cholesterol to the mitochondrial inner membrane and thus increase the neurosteroid production [70 - 73]. In minces of rat brain cortex, Ascorbic acid concentration-dependently increases the levels of pregnanolone, allotetrahydrodeoxy corticosterone.

This effect of Ascorbic acid is region-dependent; in hippocampus, dehydroepiandrosterone, progesterone and allopregnanolone are also increased, whereas dehydroepiandrosterone is unchanged; in corpus striatum only progesterone is increased significantly [66]. The full expression of the Ascorbic acid effect on neurosteroids

appears to require the presence of endogenous 5-HT, raise the possibility that Ascorbic acid modulates brain neurosteroid availability via specific 5-HT receptor subtypes positively coupled to adenylate cyclase [74 - 76], and is expressed in various rat brain regions, including cerebral cortex and hippocampus [77]. Neurosteroids are potent amplifiers of the action of GABA at GABAA receptors; exert anxiolytic, anticonvulsant, and anesthetic actions [78 - 84]. It is possible that the action of Alprazolam on GABA through the increase in neurosteroidal biosynthesis was potentiated by co-administration of Ascorbic acid, and this neurosteroids produced are potent endogenous positive modulators of the action of GABA at GABAA receptors and may related to additive anxiolytic action that was observed in this work.

Based on these lines of evidence, it appears that the anxiolytic effect of Ascorbic acid, at a dose 125mg/kg, may be due to its role in synthesis and accumulation of endogenous steroids such as allopregnanolone and pregnanolone; these endogenous steroids have an equal or greater affinity for GABA receptors than barbiturates and benzodiazepines, and modulate anxiety symptoms by facilitating GABA action at GABAA receptors [82, 84].

The anxiolytic effect of Alprazolam was not affected by Ascorbic acid. Animals treated with Ascorbic acid and Alprazolam together showed additive anxiolytic effects, which was clear with large dose of Ascorbic acid (500mg/kg). It is concluded that Alprazolam and Ascorbic acid each alone in the doses used, have anxiolytic action; the combination of Alprazolam and Ascorbic acid treatments produce additive anxiolytic effect.

Alprazolam alone or Ascorbic acid (125mg/kg) alone increase total lines and total number of entries into open and closed arms, which indicate that they increase spontaneous motor activity. The increase in spontaneous motor activity has often been described as an anxiolytic-associated increase in exploratory activity [85 - 88].

Sedation was not observed since the combined treatment did not change the total lines and entries. Ascorbic acid may acts selectively on BZ1 receptor, which is non-sedative anxiolytics [89]. The anxiolytic action of Ascorbic acid may be through potentiating endogenous BZ, which leads to increase the levels of GABA in different brain areas.

#### **Effect of acute administration of Ascorbic acid and Alprazolam on GABA levels in different brain regions**

The present data revealed that, acute administration of Alprazolam and Ascorbic acid in the dose 125 or 500mg/kg produced an increase in the GABA levels in different brain areas including whole brain.

Peter., 2005 [90], reported that, activation of GABA-benzodiazepine receptor causes an increase in the influx of chloride ions, which in turn results in the membrane

hyperpolarization that is responsible for neuronal inhibition. Benzodiazepines do not independently activate this process, but rather facilitate the action of GABA by increasing the frequency of ion channel opening [91]. In addition, benzodiazepines increase GABA synthesis through the stimulation of glutamate decarboxylation [92]. Thus, the therapeutic effects (sedation, hypnosis, and/or anxiety) of BZ might be due to the increases in GABA levels in brain, particularly in cerebellum, cerebral cortex, the limbic system and reticular formation, where the presence of the specific binding sites [26, 93]. GABA-BZ receptors are widely distributed throughout the CNS with high concentration in the prefrontal and cerebral cortex, medial temporal lobe (amygdala and hippocampus), thalamus, cerebellum, olfactory bulb and striatum [94, 95]. This action of BZ may be one apart from the well known mechanism through the full selective allosteric modulation maximizing GABA neurotransmission at the GABAA receptor [96, 97]. The modulator allosteric site on GABA-chloride channel complex mediates both facilitatory (BZ-receptor agonist) and inhibitory (inverse agonist) effects [98].

Alprazolam sharply increase GABA levels in striatum, which it was found to be the most sensitive area that showed maximum increase of GABA level with low doses of triazolam [26], and also is very sensitive to acute stress where the activity of the glutamic decarboxylase (GAD) and the levels of GABA were reduced [99]. The effect of Alprazolam on GABA concentration might be due to the fact that BZ increases GABA synthesis through the stimulation of glutamate decarboxylation [92].

In this study, the choice of different brain region for GABA levels measurement was based on the previous knowledge about different brain areas and their functions; in order to relate any possible neurochemical changes in these areas to corresponding behavioral changes. The cerebellum is an important control center for motor functions. It coordinates movement and posture and is involved in programming movements. It is known to be involved in emotional and cognitive processes [100]. The brain stem contains reticular activating system, which is essential for the regulation of sleep, wakefulness, and level of arousal, as well as for coordination of eye movements [101]. The striatum as a key position to serve as an integrating unit modulating functions in the cortex, thalamus and the limbic system [102, 103], it is very sensitive to acute stress [99]. The mid-brain with the limbic system, hypothalamus, and the hippocampus are able to influence many aspects of emotional behavior, via the hypothalamus and its connections with the outflow of the autonomic nervous system and control of the endocrine system [101, 104]. The cerebral cortex provide for supervisory integration of the autonomic nervous system, and they may integrate somatic and vegetative functions, including those of cardiovascular and gastrointestinal systems [101]. The cortex is the site

origin of all conscious and many subconscious actions; it is the collecting and processing station for sensory impressions, sensations, and perception [105].

Ascorbic acid at a doses 125 or 500mg/kg increase GABA levels in all brain regions except striatum and cerebral cortex, where Ascorbic acid 500mg/kg increase GABA level insignificantly compared to the control. Ascorbic acid have a modulating effect on glutamate function [65]. As glutamate is taken up after release, intracellular Ascorbate is released from cells by glutamate-Ascorbate heteroexchange mechanism [59, 106], this leads to influence neostriatal glutamate transmission presynaptically at the glutamate reuptake site and perhaps postsynaptically via NMDA receptor blockade [64]. The increase in brain GABA concentration might be due to the fact that, L-Ascorbic acid increase neurosteroids synthesis and accumulation in minces of various rat brain regions [66]. Neurosteroids facilitating GABA action at GABAA receptors [107].

The present results showed that, whereas Alprazolam alone increase GABA levels in striatum, while combined treatments (Alprazolam + Ascorbic acid 125, 500mg/kg) decrease it when compared to Alprazolam alone treated group, but although GABA levels still higher than the control group. The benzodiazepine-binding site is only one of multiple binding sites contained within the GABAA receptor complex, and each site modulates the effects of GABA independently [108]. McMahon and France; 2005 [109] suggested that low-efficacy benzodiazepine ligands, will effectively dose-dependently antagonize midazolam and, at the same doses, will enhance the effects of a neuroactive steroid. Within each class of modulatory compounds, the chemical configuration of a drug plays a role in determining its intrinsic efficacy [110]. The intrinsic efficacy of these modulators changes within the limits imposed by the maximal efficacy of GABA for a given GABAA receptor configuration. With this provision, the efficacy of a drug can be defined as the ratio between the number of receptors modulated by the drug and the number of receptors occupied by the drug. Because for the same degree of receptor occupancy, low-efficacy modulators (partial agonists) induce a smaller response in their target cells than do full-efficacy modulators (full agonists), one wonders about the molecular nature of their low efficacy [111]. From all the forgoing discussion and taking in consideration that, Alprazolam acts as full agonist [112], [113], it is suggested that Ascorbic acid may acts as a partial allosteric modulator of the GABAA receptors. Therefore, each alone produces an increase of GABA levels in different brain regions, leading to anxiolytic effects. The combined treatment of Alprazolam with Ascorbic acid leads to an increase in GABA levels less than the effect of each alone. The effect of Alprazolam and Ascorbic acid each alone or combined together showed the same effect on behaviour.



### **Effect of acute administration of Ascorbic acid and Alprazolam on Ascorbic acid level in different brain regions**

Data of the present work showed that, Alprazolam alone, different doses of Ascorbic acid (125 or 500 mg/kg) each alone and the combination of Alprazolam and Ascorbic acid (125 or 500 mg/kg) did not change the Ascorbate levels in cerebellum and brain stem. Brain tissue content of Ascorbate is regionally dependent: higher levels are found in anterior regions, such as the cerebral cortex and hippocampus, with progressively lower levels in more posterior regions, such as the brain stem and spinal cord. This pattern reflects the increasing white-matter content of the posterior regions of the CNS, because the ascorbate content of white matter is much lower than that of neuron-rich gray matter. Such differences may be linked to metabolic requirements of the tissue. The rate of metabolism in cortex is greater than in white matter rich brainstem and spinal cord axons, because of the higher metabolic demand of cell bodies compared with axons [114].

It has been determined that the primary mechanism leading to increases in extracellular ascorbate concentration is its heteroexchange with glutamate [59, 106, 115]. As glutamate is taken up after release, intracellular ascorbate is released from cells by a glutamate-ascorbate heteroexchange mechanism [116]. Ascorbate released during Na<sup>+</sup>-dependent, uptake of glutamate by synaptosomes from several brain regions, although not from the cerebellum [117]. Five Glutamate transporter have been identified, it is suggested that higher levels of EAAT1 (glutamate transporter) are localized in cerebellum than in other brain regions, and lower levels of EAAT2 in the cerebellum than elsewhere [118 - 120]. Given that glutamate-uptake-dependent release of ascorbate has been demonstrated in synaptosomes from forebrain regions, but not from cerebellum [117], this might implicate EAAT2 or EAAT3 rather than cerebellum – enriched EAAT1 or EAAT4, in mediating glutamate-ascorbate heteroexchange [116]. This may explain unchanged Ascorbate levels in cerebellum after Ascorbic acid administration.

In striatum, Ascorbate increased insignificantly and significantly after administration of 125mg/kg or 500mg/kg of Ascorbic acid respectively compared to the control. It was reported that, Ascorbate was found in high micromolar concentrations in extracellular fluid of mammalian striatum; this level fluctuates in response to stress, psychoactive drugs, and even behavioral state [65]. During periods of intense motor activation, striatal extracellular ascorbate can double in concentration over resting levels [115]. Extracellular ascorbate in striatum typically doubles during periods of behavioral activation [121], behavioral activation also increases striatal

glutamate release [122], further supporting an ascorbate-glutamate link.

Intrastriatal ascorbate oxidase injection caused a rapid decline in both ascorbate and behavioral activation. Within 20 minute, 50-70% loss of striatal ascorbate led to a near-total inhibition of all recorded behavior, including open-field locomotion, approach of novel objects, and social interactions with other rats [65]. There is some evidence indicating that ascorbate is released by heteroexchange at the glutamate transporter [60, 117], and that physiologically evoked release of ascorbate is inhibited by glutamate uptake inhibitors [106]. Ascorbate is present in striatal extracellular fluid at >1000 times the concentration of dopamine [123], this increasing evidence suggests that ascorbate influences synaptic function [60]. Local applications of ascorbate, for example, enhance the response of striatal neurons to iontophoresis of either dopamine or glutamate [124]. It also is interesting that glutamate transport appears to be the trigger for ascorbate release. Thus, intrastriatal infusions of glutamate promote the efflux of ascorbate into extracellular fluid [125], and this effect is blocked by inhibition of glutamate transport [126].

Special transporter appear responsible for ascorbate removal [127], indicating that extracellular ascorbate is under relatively precise control. The level of striatal ascorbate fluctuates with behavioral activity such that the highest extracellular concentration appears during peak motor behavior [121]. Striatal ascorbate release depends on the activation of glutamate-releasing afferents from the cerebral cortex [128], most likely involving heteroexchange with glutamate during glutamate uptake [129]. Thus, a change in ascorbate release reflects a change in glutamate transmission [115]. Low ascorbate release may result not from a decline in glutamate transmission, but from a failure of glutamate uptake, evidence that glutamate, which is released by corticostriatal neurons, plays a key role in striatal ascorbate release [117]. Thus, an increase in the activity of glutamatergic neurons causes an increase in extracellular ascorbate [115, 129].

The combination of Ascorbic acid (125 or 500mg/kg) with Alprazolam produced further and significant increase in the ascorbate level in striatum compared to the control and to Alprazolam alone treated group. This increase was dose dependent according to Ascorbic acid doses used. Brose et al., 1989 [130] reported that, there was a high correlation coefficient for motor activity versus release of ascorbate in striatum in unanaesthetized rats after administration of flurazepam. Alprazolam may increases glutamate levels in striatum by interfering with glutamate uptake [131], and Ascorbate will be released depending on the activation of glutamate-releasing afferents from the cerebral cortex which involving heteroexchange with glutamate during glutamate uptake [129]. Extracellular Ascorbate in striatum typically doubles during periods of

behavioral activation [121]. Behavioral activation also increases striatal glutamate release [113].

Like striatum, dorsal hippocampus has a high level of extracellular ascorbate [132]. Meile and Fillenz; 1996 [133] reported that ascorbate is regulated homeostatically and suggested that perfusion of low dose of Ascorbic acid through a microdialysis probe in rat brain caused ascorbate levels to fall, whereas higher concentrations caused an increase in ascorbate levels. This finding is supported by another study conducted by Cammack et al., 1991 [126], which shows that, hippocampal glutamate-induced ascorbate release is dose dependent and completely inhibited by the coadministration of the glutamate reuptake blockers. It is most likely that Alprazolam has no effect on Ascorbic acid action in this area, however, benzodiazepines abolished the stress-induced glutamate efflux in the prefrontal cortex and hippocampus [134], and therefore no glutamate-ascorbate heteroexchange will occur.

Ascorbate decreased in the anterior-to-posterior axis of the CNS, as reported for rat brain ascorbate [135, 136]. This pattern is consistent with the increasing white matter content of more caudal regions, because the lowest ascorbate were found in white matter and the highest in gray matter specifically, the ascorbate content of cerebral cortex was three-to fourfold greater than that in optic nerve [116]. The higher levels of Ascorbate in gray compared with white matter, for example, are consistent with higher somatic than axonal ascorbate levels. Such differences may be linked to metabolic requirements of the tissue. The rate of metabolism in cortex is greater than in white matter-rich brainstem and spinal cord axons, because of the higher metabolic demand of cell bodies compared with axons [137 - 139].

According to these findings cerebral cortex in present study showed higher Ascorbate levels compared to brain stem, Alprazolam alone did not changed the ascorbate levels in this area. Alprazolam may reduce Glutamate indirectly by potentiating GABAergic neurotransmission at GABAA receptors via a modulatory binding site, or may be due to the effect of Alprazolam on calcium ion influx that can trigger the burst firing, which lead to suppressing glutamate release from presynaptic terminals, thus glutamate Ascorbate heteroexchange, which is the result of increased glutamate levels, was not observed. Alprazolam appeared to have no effect on ascorbate levels in cerebral cortex when combined with low or high doses of Ascorbic acid.

## CONCLUSION

The aim of the study was to investigate the effect of Ascorbic acid on behaviour and on Alprazolam anxiolytic action. The main conclusion of the study was: Ascorbic acid produced dose dependent anxiolytic effects; it has no sedative effect up to the dose 500mg/kg. Alprazolam (2mg/kg), produced anxiolytic action without sedation.

The combined treatment of Ascorbic acid with Alprazolam did not potentiate the anxiolytic action, but it was additive effect. Acute administration of Ascorbic acid was accompanied by an increase in GABA levels in almost all brain areas studied, leading to the anxiolytic action. Ascorbic acid may acts as a partial allosteric modulator of the GABAA receptor, since it induces a smaller response in their target cells than do full allosteric modulator Alprazolam. Acute administration of Ascorbic acid (125 or 500mg/kg), selectively increases the Ascorbate levels in striatum, mid-brain and cerebral cortex. Alprazolam did not change Ascorbate levels in all brain areas studied.

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