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Neuroprotective Effect Of Dark Chocolate On Ketamine-Induced Animal Model Of Psychosis Through The Reversal Of Behavioral, Biochemical And Histopathological Changes

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Abstract

The present study was designed to evaluate the antipsychotic-like activity of dark chocolate against Ketamine-induced psychosis in animals and to explore the possible underlying mechanisms for this activity. Dark chocolate (200, 400 mg/kg, along with diet), haloperidol (1mg/kg, i.p.), and olanzapine (5mg/kg, i.p) were administered daily to different groups of mice and rats; after 30 min Ketamine was injected for 21 successive days. The antipsychotic- like activity was assessed using an actophotometer, stereotypic behaviors, forced swim test (FST), and passive avoidance test. Dark Chocolate (DC) and olanzapine remarkably decreased the immobility period of Ketamine-treated mice in FST, indicating the beneficial effect of DC against depressive or negative symptoms of psychosis. There was no significant effect on the locomotor activity of the mice with the administration of DC, while it showed a reducing impact against stereotypic behaviors. Furthermore, DC significantly reduced the time taken by the rats to climb the pole, showing the effect of DC on cognition. In addition, acetylcholinesterase (AChE) activity, malondialdehyde (MDA), and tissue necrosis factor (TNF-α) levels were markedly decreased and increased GABA and enhanced reduced glutathione (GSH) levels with the treatment of DC. Furthermore, DC was also effective in reducing the ketamine induced hyperchromatic nuclei, perinuclear vacuolization, and dilated vascular channels in the cortex part of the brain. Behavioral, biochemical, and histopathological studies suggest the protective effect of DC against Ketamine induced psychosis.

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Keypoints: Psychosis, dark chocolate, dopamine, GABA, GSH

INTRODUCTION

Psychosis is a chronic neuropsychiatric disorder that occurs due to oxidative stress, neurotransmitter imbalance (dopamine, GABA, serotonin, acetylcholine, glutamate), neuroinflammation, and mitochondrial dysfunction (Kumar et al., 2017). Individuals affected with psychosis are prone to aggression, stereotypic behaviors, suicidal thoughts, anxiety, depression, and cognitive impairment (Mullen, 2006). Currently, typical and atypical antipsychotics are used to treat the various symptoms of psychosis. However, these antipsychotic agents are associated with serious side effects, incomplete efficacy, and increased risk of psychotic relapse (Fell et al., 2012). Apart from these, metabolic syndrome and extrapyramidal symptoms are the most prominent reasons that limit the clinical use of these antipsychotic agents. Moreover, these drugs fail to reverse this disorder and provide only symptomatic relief (Baldessarini, 2001) . Therefore, overall brain function and quality of life of psychotic patients remain poor after treatment with these drugs (Ray et al., 2009; Yadav et al., 2021). Consumption of diets enriched with phytonutrients or bioactive constituents like antioxidants, anti-inflammatory agents, neuroprotective, etc., may provide favorable effects to alleviate psychiatric complications with no side effects (Yadav et al., 2016). Besides, dietary supplements or natural products structurally hold biochemical specificity with broad spectrum chemical diversity (Chatterjee et al., 2011). Thus, a more valuable approach for treating psychotic patients could be nutritional therapy involving natural products. Thus, we have selected the most delicious product of cocoa or cacao, that is, dark chocolate (DC), to screen its potential effect on psychosis. Dark chocolate is a functional food prepared from cacao beans. Cacao is a dry, powdered product prepared from the seeds of the *Theobroma cacao* L. belonging to the family Sterculiaceae; cacao beans or cacao seeds are found in Cacao pods. Cacao contains plenty of polyphenols, flavonoids, neuroactive compounds, phytonutrients, etc., so it is considered a "Super Food" (Fisher et al., 2006; Alexander et al., 2013). These chemical constituents possess neuroprotective, anti- inflammatory,anti-Alzheimer's, and antioxidant effects (Hollenberg et al., 2009; Alexander et al., 2013). Therefore, we hypothesized dark chocolate may be helpful in the management of psychosis and allied disorders.

MATERIALS AND METHODS

Experimental animals

Swiss albino mice of either sex, weighing 20-25g, and Wistar albino male rats, weighing 150–200 g, were procured from Disease Free Small Animal House, LalaLajpatRai University of Veterinary and Animal Sciences, Hisar (Haryana, India). The animals were housed separately in groups of 6 per cage under standard laboratory conditions with alternating light and dark cycles of 12 h each. The animals had free access to food and water. The animals were acclimatized for seven days before the start of experiments. Institutional Animals Ethics Committee approved the experimental protocol and animal care was taken per the Committee's guidelines for the Purpose of Control and Supervision of Experiments on Animals, Government of India (Registration no. 0436).

Selection of dietary nutrient

Dark Chocolate (Amul, India), containing 56% cocoa, was used for the present study.

Drugs and Chemicals

Olanzapine (Intas Pharmaceuticals Pvt. Ltd., India), haloperidol (RPG Science Pharmaceutical Pvt. Ltd., India) and ketamine (Neon Pharmaceutical Pvt. Ltd., India) were used in the present study. Olanzapine, haloperidol and ketamine were diluted separately in normal saline. All drug solutions were freshly prepared and administered to animals. All other chemicals used for biochemical estimations were of analytical reagent grade.

Selection of doses

The doses of haloperidol (1mg/kg, i.p.), olanzapine (5mg/kg, i.p.), ketamine (50mg/kg, i.p.) and dark chocolate (200mg/kg and 400mg/kg, along with diet) were selected based on literature (Astrid et al., 2012; Yadav et al., 2017).

Experimental Protocol

Sub-chronic and chronic administration of ketamine (50 mg/kg, i.p.) induces three types of psychotic symptoms: positive, negative and cognitive symptoms (Kumar et al., 2017). Animal models having potential similarities to signs and symptoms of psychosis were used in the present study. On the 7th day of drug administration, behavioral tests were carried out for locomotor activity (a positive symptom of psychosis). On the 10th day, stereotypic behaviors were assessed just after the administration of ketamine. Further, on the 14th day of drug administration, a forced swim test was used to assess anhedonia, negative symptoms of psychosis. Cognitive impairment used to measure memory and the learning effect of antipsychotic agents were assessed using a pole climbing avoidance test on the 20th day of drug administration. On the 21st day, a bar test was performed to screen the cataleptic effect of dark chocolate, and afterward, biochemical estimations were done.

Group 1 and 2: Vehicle-treated mice and rats, respectively
Group 3 and 4: Ketamine (50 mg/kg, i.p.) treated mice and rats, respectively
Group 5 and 6: Haloperidol (1 mg/kg, i.p.) treated mice and rats, respectively, ketamine (50 mg/kg, i.p.) was injected after 30 minutes of haloperidol administration
Group 7 and 8: Olanzapine (5 mg/kg, i.p.) treated mice and rats, respectively, ketamine (50 mg/kg, i.p.) was injected after 30 minutes of olanzapine administration
Group 9 and 10: DC (200 and 400 mg/kg, along with diet, respectively) was administered to mice, ketamine (50 mg/kg, i.p.) was injected after 30 minutes of DC administration
Group 11 and 12: DC (200 and 400 mg/kg, along with diet, respectively) was administered to rats, ketamine (50 mg/kg, i.p.) was injected after 30 minutes of DC administration
Mice were used to estimate the level of TNF-α, GABA, and dopamine in the brain, whereas rats were used to estimate the level of GSH, MDA, total protein content, and AChE activity in the animal brains. Separate mice were used for the histopathological studies.

Ketamine-induced hyper locomotor activity in mice
Sub-chronic administration of ketamine (50mg/kg, i.p.) produced hyperlocomotor activity in mice, which was counted by actophotometer (INCO, Ambala, India). Mice were placed individually in actophometer chamber for 5 minutes as a habituation period before recording total locomotor count.Total number of locomotor scores was counted for next 5 minutes(da Silva et al., 2010).

Ketamine-induced stereotypic behaviors in mice
Sub-chronic administration of ketamine (50mg/kg, i.p.) induced stereotypic behaviors in mice, assessed by the number of falling (falls), turning (turn around), weaving (pacing to and fro over the same point, with frequent rears when turning) and head bobbing (neck waving right and left and going up and down) for 10 minutes after 30 minutes of administration of inducer (Kumar et al., 2022).

Ketamine-induced stereotypic behaviors in mice
Chronic administration of ketamine (50mg/kg, i.p.) in mice, able to induce immobility, a sign of depression or negative symptom of psychosis-like anhedonia, was assessed by forced swim test (Chatterjee et al., 2011). Mice were individually forced to swim in a tub filled with water up to a height of 15 cm. After 2 minutes of initial vigorous activity, mice showed immobility by floating with minimal movements. A mouse is considered to be immobile when it remains floating passively in water in a slightly hunched but upright position with its nose above water. The total immobility period during the 4 minutes of the test was recorded (Porsolt et al., 1977).

Ketamine-induced cognitive symptoms of psychosis in rats
This model is used to evaluate the effect of newer drugs on cognitive symptoms. The apparatus consists of an attenuated chamber (25×25×40 cm^3) having a grid floor with a wooden pole at its center. The 28 V light and 2.8 kHz speaker were fixed at the chamber ceiling for conditioned stimulus (4 seconds), and 1.5 mA electric shocks were delivered to the floor (for the next 26 seconds) for unconditioned stimulus. The training was given to climb the pole to avoid shocks. The learning session comprised 20 trials at the interval of 90 seconds. Memory session was performed after 24 hours of the learning session by providing conditioned stimuli only. Pole climb response during the conditioned stimuli is considered an avoidance response, and the time taken by the animals to climb the pole after giving the conditioned stimuli was noted for a maximum period of 90 seconds (Sharma et al., 2016).
Bar test of catalepsy in mice

The bar test has been used for the measurement of cataleptic side effects of antipsychotic drugs. On the 21st day of the protocol, the mice were placed with both front paws on a horizontal bar, which was at 4.5 cm height and parallel to the base. The time was noted till the animal removed one paw from the bar. The maximum cut-off time for the bar test was 180 seconds (Chatterjee et al., 2012).

Biochemical estimations

Collection of blood samples for the estimation of TNF-αlevel

After behavioral assessments, blood was withdrawn from the retro-orbital plexus site and centrifuged for 15 minutes at 3000 rpm (Remi Instruments, India) to separate serum, which was used further to measure TNF-α level by ELISA technique. Briefly, 50 µl of assay diluents were added into pre-coated plate wells, and 50µl of the test or standard sample was mixed, covered with strip, and placed for incubation at 37 ˚C for 2 hours. Wells were washed and aspirated five times with buffer, and 100 µl of animal TNF-Conjugate was added and incubated at room temperature for 2 hours. Again, washings and aspiration, 100 µl substrate solution was added into the wells and incubated at room temperature for 30 minutes. Finally, 100 µl stop solution was added into each well and absorbance was recorded at 450 nm using ELISA reader (Erba, Germany).

Dissection and isolation of the brain

After the collection of blood, animals were sacrificed using the decapitation method; brains were isolated, washed, weighed, homogenized, and used for the estimation of biochemical parameters.

Estimation of total protein

0.1 M phosphate buffer (pH 7.4) was prepared to homogenate brain tissue and used to estimate total brain protein content by biuret method using bovine serum albumin as standard (Gornall et al., 1949).

Estimation of reduced glutathione (GSH) level

GSH level was estimated as per the method described by Ellman, 1959. 0.1 M phosphate buffer (pH 7.4) was used to homogenate the brain tissue, and 1 ml homogenate was precipitated by adding 1 ml trichloroacetic acid (TCA) and centrifuged for 10 minutes at 500 rpm. 0.5 ml brain supernatant was taken and mixed with 2 ml disodium hydrogen phosphate buffer (0.3 M, pH 8.4) and double distilled water (0.4 ml). Then, 0.001 M DTNB [5,5-dithio- bis-(2-nitrobenzoic acid)] (0.25 ml) was added into the above mixture and kept for incubation at room temperature for 10 minutes. Yellow colour so developed was recorded at 412 nm by UV-Visible spectrophotometer (Varian Cary 5000, Netherland) and results were calculated using molar extinction coefficient 1.36×10^4 $M^{-1}cm^{-1}$ and represented as µmol/mg of protein (Ellman, 1959).

Estimation of malondialdehyde (MDA) level

The degree of lipid peroxidation was quantified by estimating the MDA level in the form of thiobarbituric acid reactive substances (TBARS). 0.1 M phosphate buffer (pH 7.4) (1 ml) was used to homogenate the brain tissue and 0.5 ml of homogenate was added into 0.5 ml of Tris- HCl followed by incubation at 37 ºC for 2 hours. TCA(1 ml) was added to the above mixture and centrifuged at 1000 rpm for 10 minutes. Then, supernatant (1 ml) was withdrawn, added into 0.67% thiobarbituric acid (1 ml), and boiled for 10 minutes. After cooling, double distilled water (1 ml) was added, and absorbance was taken at 532 nm by UV-visible spectrophotometer. TBARS was estimated using extinction coefficient (1.56×10^5 $M^{-1}cm^{-1}$) and the data obtained is represented as nmol/mg of protein (Wills, 1964).

Estimation of acetylcholinesterase (AChE) activity

0.01 M sodium phosphate buffer (pH 8) was used to homogenate brain tissue, followed by centrifugation at 3000 rpm for 10 minutes. 0.01 M sodium phosphate buffer (2.6 ml, pH 8) and DTNB (0.1 ml) were mixed with supernatant (0.4 ml), and absorbance was taken at 412 nm by UV-visible spectrophotometer. Then, acetylthiocholine iodide (0.02 ml) was added to the above mixture, stood for 15 minutes, and absorbance was again taken at 412 nm. Change in absorbance was calculated; results were calculated by molar extinction coefficient (1.36×10^4 $M^{-1}cm^{-1}$) and showed as nmol/minute/g of brain weight (Ellman et al., 1961).

Estimation of gamma-aminobutyric acid (GABA) level

Brain tissue was homogenized in 0.01 M HCl (5 ml). 0.1 ml of homogenate was added to 0.14 M ninhydrin solution (0.2 ml) made in 0.5 M carbonate bicarbonate buffer (pH 9.95), followed by heating at 60 °C for 30 minutes. After cooling, 5 ml of copper tartrate reagent (0.03% copper sulphate, 0.16% sodium carbonate and 0.0329% tartaric acid) was added, kept for 10 minutes and absorbance was noted at 377/455

nm by spectrofluorimeter (Model 152, Systronic, India) (Lowe et al., 1958).

Estimation of dopamine level

Brain tissue was homogenized in 3 ml of HCl-butanol (0.1M HCl in butanol) and centrifuged at 2000 rpm for 10 minutes. 0.8 ml of supernatant was added into 0.25 ml of 0.1 M HCl and 2 ml of heptane, followed by shaking for 10 minutes and centrifugation. 0.02 ml of aqueous phase was separated and added to 0.05 ml of 0.4M EDTA and 0.01 ml of sodium acetate buffer of pH

6.9. After that, 0.01 ml iodine solution (0.1M in ethanol) was added for oxidation for 2 minutes, and 5M sodium thiosulphate (0.5 ml) was used to stop the reaction. After 90 seconds, 10M acetic acid (0.5 ml) was added, boiled for 6 minutes, cooled, and analyzed at 330/375 nm by spectrofluorimeter. Tissue values (fluorescence of tissue extract less fluorescence of tissue blank) were compared with internal reagent standard (fluorescence of internal reagent standard less fluorescence of internal reagent blank). Tissue blank was prepared by the addition of reagents of the oxidation step in reverse order, i.e., sodium thiosulphate before iodine while keeping all other materials constant. For the preparation of the internal reagent blank, 0.125 ml distilled water was added into 2.5 ml HCl-butanol (0.1 M HCl in butanol), while the internal reagent standard was prepared by adding 0.125 ml distilled water, 2.5 ml HCl-butanol (0.1 M HCl in butanol) into 500 ng dopamine (Schlumpf et al., 1974).

Histopathological studies

Brains were preserved in 10% formalin and paraffin sectionswere cut and stained with hematoxylin and eosin for histopathological studies.

Statistical analysis

All the results were expressed as mean ± SEM. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests using Graph Pad Instat (San Digeco, USA). $p<0.05$ was considered statistically significant.

RESULTS

Effect of DC on Ketamine induced hyper locomotor activity in mice

Locomotor activity was increased by administration of ketamine (50 mg/kg, i.p.) for 7 successive days. DC (200 and 400 mg/kg, along with diet) did not produce any effect on ketamine induced hyperlocomotor activity. Haloperidol (1 mg/kg, i.p.) and olanzapine (5 mg/kg, i.p.) showed a decrease in locomotor activity as compared to ketamine-treated mice (Figure 1).

Effect of DC on Ketamine induced stereotypic behaviors in mice

Stereotypic behaviors were induced by the administration of ketamine (50 mg/kg, i.p.) for 10 successive days. DC (400 mg/kg, along with diet) significantly [$F(5, 30) = 29.889$, ($p<0.05$)] reduced ketamine-induced stereotypic behaviors. Haloperidol (1 mg/kg, i.p.) and olanzapine (5 mg/kg, i.p.) also showed a reduction in stereotypic behaviors as compared to ketamine- treated mice (Figure 2).

Effect of DC on Ketamine-induced immobility in mice

Immobility duration was increased by the administration of ketamine (50 mg/kg, i.p.) for 14 successful days. DC (200 and 400 mg/kg, along with diet) significantly [$F(5, 30) = 51.37$, ($p<0.001$ and $p<0.001$, respectively)] decreased ketamine increased immobility. Olanzapine (5 mg/kg, i.p.) also showed a decrease in immobility duration as compared to ketamine- treated mice(Figure 3).

Effect of DC on Ketamine induced cognitive deficits in rats

Latency to climb the pole was increased by the administration of ketamine (50 mg/kg, i.p.) for 20 successive days. DC (200 and 400 mg/kg, along with diet) significantly [$F(5, 30) = 47.487$, ($p<0.001$ and $p<0.001$, respectively)] decreased the latency to climb the pole. Olanzapine (5 mg/kg, i.p.) also decreased the latency to climb the pole as compared to ketamine-treated rats (Figure 4).

Effect of DC on catalepsy in mice

Cataleptic effect was observed with the haloperidol (1 mg/kg, i.p.) treated mice as compared to the vehicle-treated group. In contrast, DC (200 and 400 mg/kg, along with diet) did not show any cataleptic effect (Figure 5).

Effect of DC on Brain Total Protein Content

Ketamine (50 mg/kg, i.p.) depleted the total protein content in the brain compared to the vehicle-treated group. DC (200 and 400mg/kg, along with diet) showed significant [$F (5, 30) = 167.76$, ($p<0.001$ and $p<0.001$, respectively)] increase in the brain protein content. Haloperidol (1 mg/kg, i.p.) and olanzapine (5 mg/kg, i.p.) were also found to restore the brain protein content as compared to ketamine-treated rats (Table 1).

Effect of DC on serum TNF-α level

Ketamine (50 mg/kg, i.p.) increased serum TNF-α levels as compared to vehicle-treated mice. DC(200and400mg/kg, along with diet)significantly [$F (5, 30) = 102.73$, ($p<0.001$ & $p<0.001$, respectively)] diminished the serum TNF-α level. Haloperidol (1 mg/kg, i.p.) and olanzapine (5 mg/kg, i.p.) were also found to decrease ($p<0.001$ & $p<0.001$, respectively) the serum TNF-α level as compared to ketamine treated group (Table 1).

Effect of DC on brain GSH level

Ketamine (50 mg/kg, i.p.) decreased the brain GSH level compared to the vehicle-treated group. DC (200 and 400mg/kg, along with diet) showed significant [$F (5, 30) = 19.721$, ($p<0.05$ and $p<0.001$, respectively)] increase in the brain GSH level. Haloperidol (1 mg/kg, i.p.) and olanzapine (5 mg/kg, i.p.) were also found to increase the brain GSH level as compared to ketamine-treated rats (Table 1).

Effect of DC on brain MDA level

Ketamine (50 mg/kg, i.p.) increased the lipid peroxidation by increasing the MDA level as compared to vehicle treated group. DC (200 and 400mg/kg, along with diet) showed significant [$F (5, 30) = 18.852$, ($p<0.001$ and $p<0.001$, respectively)] decrease in MDA level.

Haloperidol (1 mg/kg, i.p.) and olanzapine (5 mg/kg, i.p.) were also found to decrease brain MDA levels as compared to ketamine-treated rats (Table 1).

Effect of DC on brain AChE activity

Ketamine (50 mg/kg, i.p.) increased the brain AChE activity as compared to vehicle treated group. DC (200 and 400mg/kg, along with diet) significantly [$F (5, 30) = 16.385$, ($p<0.01$ and $p<0.001$, respectively)] decreased the activity of AChE in rats brain. Haloperidol (1 mg/kg, i.p.) and olanzapine (5 mg/kg, i.p.) were also found to decrease the brain activity of AChE as compared to ketamine-treated rats (Table 2).

Effect of DC on brain GABA level

Ketamine (50 mg/kg, i.p.) reduced the brain's GABA level as compared to the vehicle-treated group. DC (200 and 400mg/kg, along with diet) showed a significant [$F (5, 30) = 52.173$, ($p<0.001$ & $p<0.001$, respectively)] increase in GABA level. Haloperidol (1 mg/kg, i.p.) and olanzapine (5 mg/kg, i.p.) were also found to increase the brain GABA level as compared to ketamine-treated mice (Table 2).

Effect of DC on brain dopamine level

Ketamine (50 mg/kg, i.p.) increased the brain dopamine level compared to the vehicle-treated group. DC (200 and 400mg/kg, along with diet) did not produce any significant effect [$F (5, 30) = 109.53$]. At the same time, haloperidol (1 mg/kg, i.p.) and olanzapine (5 mg/kg, i.p.) were effective in decreasing the brain dopamine level as compared to ketamine-treated mice (Table 2).

Effect of DC on histopathology of mice brain

Ketamine-treated animals showed hyperchromatic nuclei, perinuclear vacuolization, and dilated vascular channels in the cortex part of the brain as compared to vehicle-treated mice, whereas DC showed a reversal in these histopathological changes in the cortex region of the brain (Picture 1).

DISCUSSION

Oxidative damage, neurotransmitter imbalance, neuroinflammation, etc., are the initiative events that diverge into multiple neuropsychiatric disorders, including psychosis (Yadav et al., 2016). Consumption of dietary nutrients enriched with vital phytochemicals may provide beneficial effects in alleviating psychiatric disorders through their anti- inflammatory, antioxidant, neuroprotective effects, etc. (Meydani et al., 1998;

Yadav et al., 2016). Therefore, we have selected a highly consumed nutrient, viz. dark chocolate, for its huge medicinal value. The current study aimed to assess the effect of DC against ketamine- induced psychosis in small laboratory animals. Animal models used for screening antipsychotic drugs are based on the neurotransmitter hypothesis of psychosis, involving mainly dopamine, GABA, and acetylcholine (Lipska, 2000).

Pharmacological and clinical studies have indicated the role of the N-methyl-D- aspartate (NMDA) receptor in the pathophysiology of psychosis (Paola et al., 2011). Ketamine is an NMDA receptor antagonist that is able to induce psychosis-like symptoms in small animals as well as humans (Chatterjee et al., 2011). In our findings, DC was unable to reduce ketamine-induced hyperactivity in mice; however, it showed a significant decrease in ketamine-induced stereotypic behaviors. The stimulation of locomotor activity has been mainly attributed to the dopaminergic hyperactivation in the striatal area of the animal brain (Bennett M, 2009; Chatterjee et al., 2012). It has also been reported that dopamine neurotransmission is involved in the motor-activating effects of NMDA. In biochemical estimations, DC did not show any significant effect on dopamine levels but increased GABA levels. Both behavioral and biochemical studies collectively indicate that DC is only effective in stereotypic behaviors, but it fails to decrease dopamine. It is ineffective against locomotor activity. Neuropeptide, catechin, epicatechin, stigmasterol, gallic acid, quercetin derivatives and epigallocatechin are the main constituents found in dark chocolate, which have been reported to possess GABA potentiating effect(Binder et al., 2001; Hanrahan et al., 2011). In addition, DC was also screened for extra-pyramidal side effects using bar test of catalepsy, which is shown by haloperidol (typical antipsychotic drugs). DC (200mg/kg and 400mg/kg) treated mice did not show any cataleptic effect in bar test.

Chronic treatment with ketamine increased the immobility period used to test negative symptoms of psychosis in mice (Bennett, 2009; Chatterjee et al., 2012). In our study, DC administration showed decreased immobility periods, indicating the beneficial effect of DC against negative symptoms of psychosis in mice. Furthermore, dopamine metabolism produces free radicals, which may elevate negative and cognitive symptoms of psychosis.

Also, oxidative damage of fatty acids by inactivation of membrane-bound enzymes can cause lipid peroxidation, which may involve psychotic symptoms (Asevedo et al., 2013). MDA, a lipid peroxidation product, can be used to estimate the lipid peroxidation level (Siddique et al., 2012). Glutathione (GSH) is an endogenous free radical scavenger and protects cellular components from further oxidative damage (Kumar et al., 2014). It has been reported that increased GSH levels and decreased MDA levels in the brain are responsible for neuroprotective effects. Oxidative stress is well known to produce neuroinflammation and vice-versa (Agostinho et al. 2010). In addition, oxidative stress causes microglial cell activation that increases the release of inflammatory cytokines, which further reinforces oxidative stress, leading to neuronal toxicity and the progression of psychosis (Monji et al. 2009; Lull and Block 2010; Laskaris et al. 2016). In the present study, it has been observed that the administration of ketamine impaired the antioxidant system in the brain, which was reversed with the treatment of dark chocolate. DC administration markedly increased brain glutathione levels and decreased MDA levels, suggesting the scavenging of free radicals and the neuroprotective effect of DC. Furthermore, DC also effectively reduced TNF- α levels in animal serum, reflecting its anti-inflammatory effect. These effects might be mediated through its phytonutrients and bioactive constituents such as caffeine, theobromine, theophylline, stigmasterol, gallic acid, catechin, epicatechin, quercetin derivatives, epigallocatechin and vitamins, which are reported to have antioxidant, anti-inflammatory and neuroprotective effects (Alexander et al., 2013; Mansouri et al., 2013; Chhillar and Dhingra, 2013, Pratiwi et al., 2021, Ikram et al., 2020, Bhat and Kumar, 2022). These effects point to the direct role of DC in the management of mental disorders.

Pole climb avoidance test mainly affects cognitive behaviors through the mesocortical pathway of dopaminergic neurons in rats' brains (Chandel et al., 2012). It is also used to separate antipsychotics from sedatives and anxiolytics (Sharma et al., 2016). As it has been reported that sedative compounds suppress both avoidance and escape responding at around the same doses, antipsychotics reduce avoidance responding at lower doses than those affecting escape responding (Kumar et al., 2017). In the present study, DC significantly delayed the latency time taken by the rats to climb the pole, indicating cognitive improvement and the neuroleptic effect of DC. Acetylcholine regulates the cognitive functions in the brain, which is degraded by the enzyme acetylcholinesterase (Samochocki et al., 2003; Jakubík and El-Fakahany, 2010). DC was successful to decrease the AChE activity, which revealed the beneficial effect of DC on cognitive symptoms of psychosis. It has been reported that caffeine, stigmasterol, gallic acid, catechin, epicatechin, quercetin derivatives,

epigallocatechin, and vitamins have the potential to promote memory and cognitive function by protecting the neurons from neurotoxins, neuroinflammation, and decrease AChE activity in the brain. These mechanisms also seem to be involved in the present study, thereby improving the cognitive function of rats.

CONCLUSION

The present study shows that DC was effective against ketamine-induced hyperactivity, stereotypic behaviors, depressive or negative symptoms, and cognitive symptoms in rodents. No cataleptic effects were observed with the administration of DC, which are usually found with the use of typical antipsychotic drugs. Moreover, DC was effective in increasing the brain GABA and GSH levels while reducing the MDAand TNF-α levels and AChE activity in psychotic animals. Both behavioral and biochemical investigations suggest the antipsychotic effect of DC. These observations of DC on laboratory models provide a clue for investigating novel antipsychotics in natural substances. In nutshell, DC deserves clinical evaluation for its beneficial effects on psychotic symptoms.

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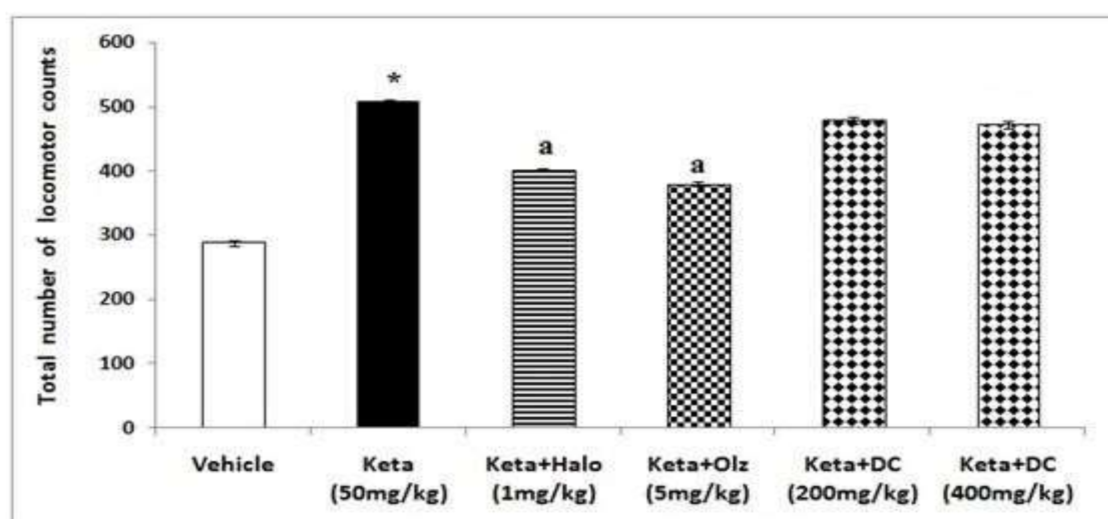


Figure 1: Effect of DC on Ketamine-induced hyper locomotor activity in mice
Values are expressed as mean ± SEM, (n = 6). Data was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. '*' p<0.001 vs. Vehicle group, 'a' p<0.001 vs. Ketamine group

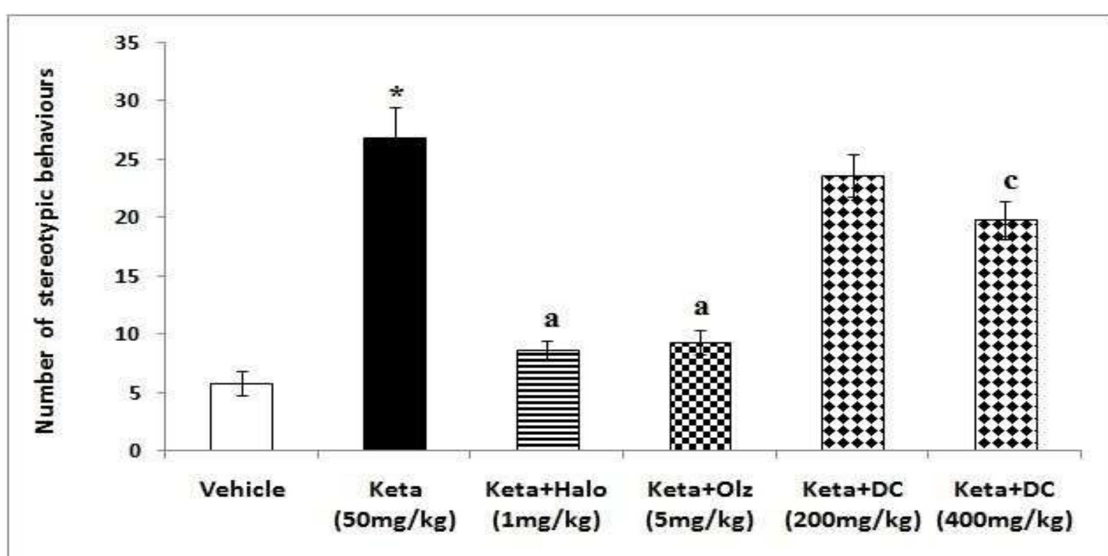


Figure 2: Effect of DC on Ketamine-induced stereotypic behaviour in mice
Values are expressed as mean ± SEM, (n = 6). '*' p<0.001 vs. Vehicle group, 'a' p<0.001 and 'c' p<0.05 vs. Ketamine group.

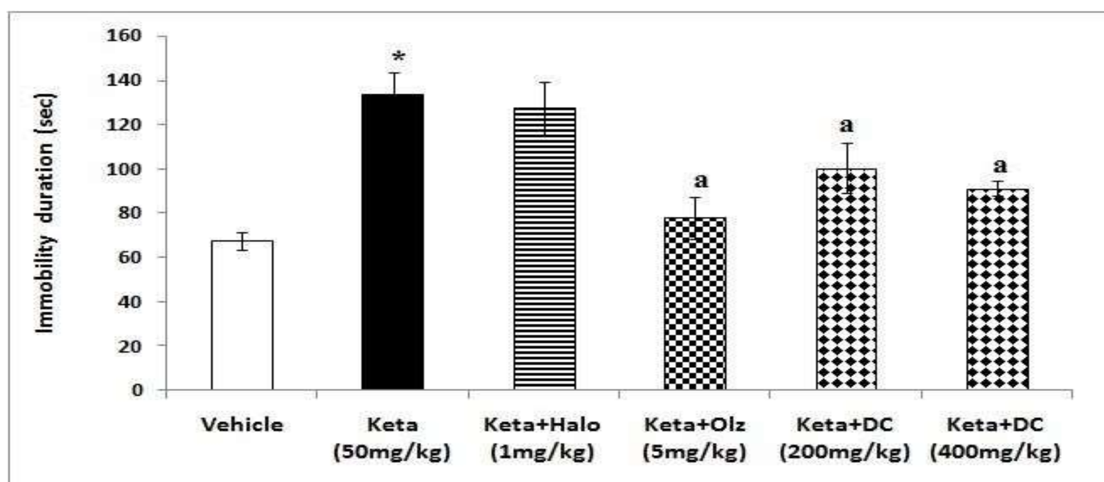


Figure 3: Effect of DC on Ketamine-induced immobility period in mice
Values are expressed as mean ± SEM, (n = 6). '*' p<0.001 vs. Vehicle group, 'a' p<0.001 vs. Ketamine group.

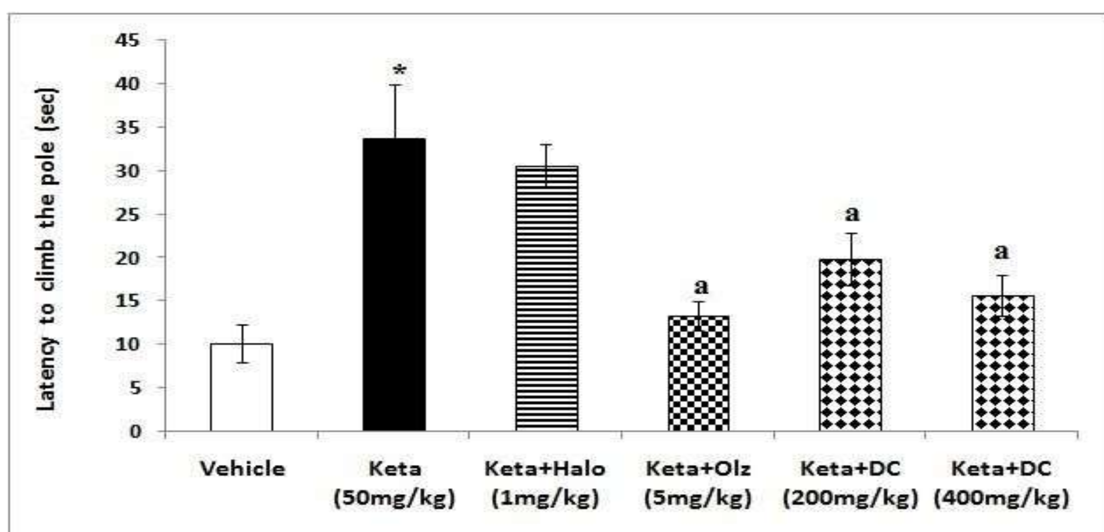


Figure 4: Effect of DC on Ketamine-induced cognitive deficits in rats
Values are expressed as mean ± SEM, (n = 6). '*' p<0.001 vs. Vehicle group, 'a' p<0.001 vs. Ketamine group.

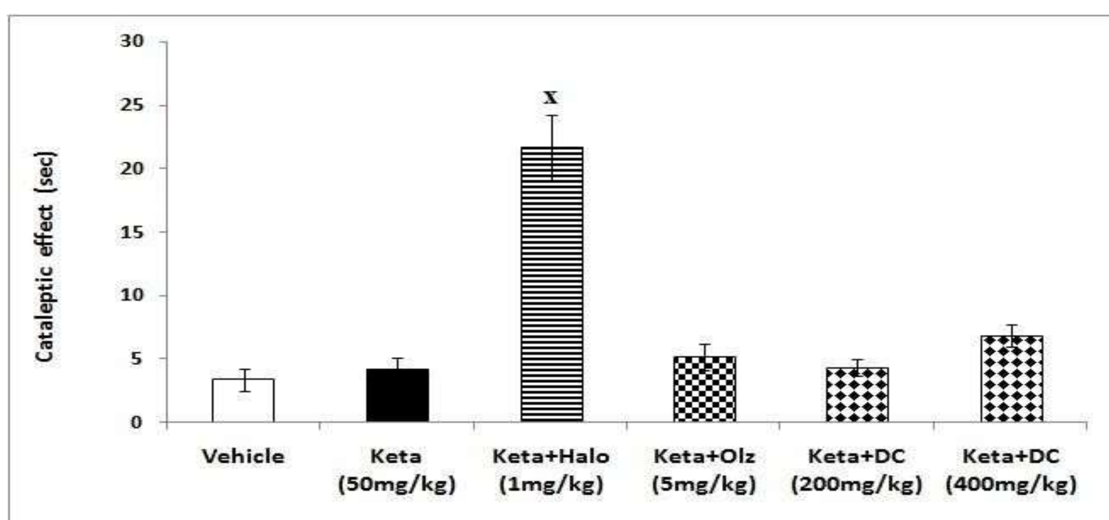


Figure 5: Effect of DC on catalepsy in mice
Values are Mean ± SEM (n = 6). One-way ANOVA followed by Tukey's test. 'x' p<0.001 vs. Vehicle group

Table: 1 Effect of Dark chocolate (DC) on total protein content, TNF-α, GSH and MDA levels

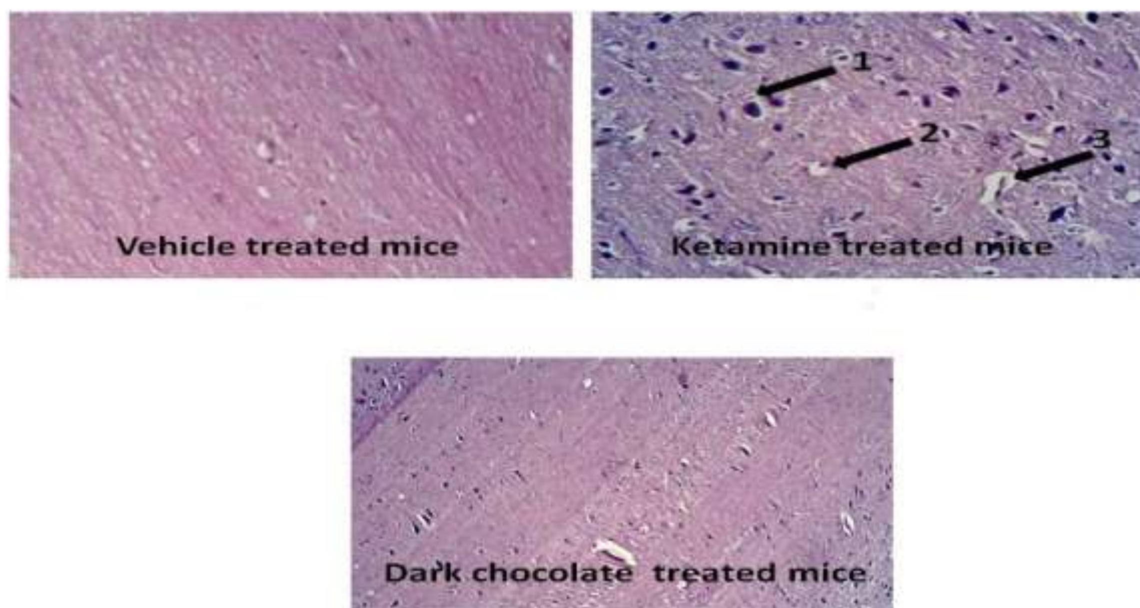
Treatment groups	Total protein content (mg/ml)	TNF-α level (ng/mL)	GSH level (μmol/mg protein)	MDA level (nmol/mg protein)
Vehicle	0.28 ± 0.005	198.83 ± 9.01	0.0048±0.0003	0.22 ±0.015
Keta (50mg/kg)	0.13±0.003*	576.66±14.39*	0.0022±0.0001*	0.48 ±0.039 *
Keta + Halo (1mg/kg)	0.23±0.001 [a]	314.33.±10.28[a]	0.0041±0.0002[a]	0.27 ±0.021 [a]
Keta + Olz (5mg/kg)	0.27±0.009 [a]	291.33±14.38[a]	0.0044±0.0002[a]	0.24 ±0.012 [a]
Keta + DC (200 mg/kg)	0.18±0.001 [a]	456.16±14.49[a]	0.0031±1.900[c]	0.31 ±0.014 [a]
Keta + DC (400mg/kg)	0.26±0.003 [a]	345.66±11.02[a]	0.0039±0.0001[a]	0.26 ±0.015 [a]

Values are expressed as mean ± SEM, (n = 6). '*' p<0.001 vs. Vehicle group, 'a' p<0.001, 'c' p<0.05 vs. Ketamine group.

Table: 2 Effect of Dark chocolate (DC) on AChE activity, GABA and dopamine levels

Treatment groups	AChE activity (nmol/min/g tissue)	GABA level(μg/g brain)	Dopamine level (pg/mg brain)
Vehicle	33.89±1.9	256.11±2.59	439.11 ±36.11
Keta (50mg/kg)	56.01±3.18*	136.77±1.56*	965.44±23.41*
Keta+Halo (1mg/kg)	34.98±2.11[a]	258.33±3.39[a]	491.78±21.32[a]
Keta+Olz (5mg/kg)	32.33±1.32[a]	259.66±1.60[a]	504.60±18.23[a]
Keta+DC (200mg/kg)	44.16±1.33[b]	193.83±9.99[a]	908.13 ±16.11
Keta+DC (400mg/kg)	36.66±2.83[a]	218.66±12.33[a]	890.95 ±19.66

Values are expressed as mean ± SEM, (n = 6). '*' p<0.001 vs. Vehicle group, 'a' p<0.001 and 'b' p<0.01 vs. Ketamine group.



Picture 1: Effect of DC on histopathological changes in brain cortex region

1: Hyperchromatic Nuclei, 2: Perinuclear Vacuolization, 3: Dilated Vascular Channels In Cortex Part Of Brain

CONFLICT OF INTEREST

There is no conflict of interest.