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Containment of neuroimmune challenge by diosgenin confers amelioration of neurochemical and neurotrophic dysfunctions in ketamine-induced schizophrenia in mice

Benneth Ben-Azu [a,b,*], Olusegun G. Adebayo [a,c], Aliance R. Fokoua [a,d], Jackson E. Onuelu [a], Jerome N. Asiwe [e], Emuesiri G. Moke [a], Itivere A. Omogbiya [a], Oghenemarho L. Okpara [a], Jennifer E. Okoro [a], Omadevuaye M. Oghenevwerutevwe [a], Christian I. Uruaka [f]

[a] DELSU Joint Canada-Israel Neuroscience and Biopsychiatry Laboratory, Department of Pharmacology, Faculty of Basic Medical Sciences, College of Health Sciences, Delta State University, Abraka, Delta State, Nigeria
[b] Division of Medical Sciences, University of Victoria, Canada
[c] Neurophysiology Unit, Department of Physiology, Faculty of Basic Medical Sciences, University of Ibadan, Ibadan, Oyo State, Nigeria
[d] Research Unit of Neuroinflammatory and Cardiovascular Pharmacology, Department of Animal Biology, Faculty of Sciences, University of Dschang, Cameroon
[e] Department of Physiology, Faculty of Basic Medical Sciences, College of Health Sciences, Delta State University, Abraka, Delta State, Nigeria
[f] Department of Pharmacology and Therapeutics, Faculty of Basic Medical Sciences, College of Medicine, Port Harcourt, Rivers State University, Port harcourt, Rivers State, Nigeria

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ABSTRACT

Inhibition of neuroinflammation through N-methyl-D-aspartate receptor (NMDAR) regulation can provide normalization of neurochemical homeostasis and neurotrophic support in the pathogenesis of psychiatric disorders with complex symptoms such as schizophrenia. Accordingly, the preventive and reversal effects, and potential mechanisms of diosgenin, a phyto-steroidal sapogenin with anti-inflammatory functions, was evaluated in ketamine (an NMDAR antagonist) model of schizophrenia in mice. Adult male mice were allotted into 5 groups. In the preventive protocol, mice received saline (10 mL/kg), diosgenin (25 and 50 mg/kg) and risperidone (0.5 mg/kg) orally for 14 days, with additional injection of ketamine (20 mg/kg/day/i.p.) from days 8–14. In the reversal protocol, mice took ketamine injection consecutively for 14 days prior to diosgenin and risperidone treatments from days 8–14. Thereafter, schizophrenia-like behavior, therapeutic extrapyramidal adverse effect, neuroimmune, neurochemical and neurotrophic consequences in important brain areas affected in the disorder were assayed. Diosgenin prevented and reversed stereotypy behavior, cognitive impairment, and psychotic-depression relative to ketamine groups. Complementarily, diosgenin prevents and reverses ketamine-induced dopamine and serotonin alterations in the striatum, prefrontal-cortex, and hippocampus relative to ketamine groups. Except for the cortical regions, diosgenin prevented and reversed glutamic acid decarboxylase depletion in these brain regions by ketamine, suggesting improved GABAergic system. Additionally, ketamine-induced elevation of neuroinflammatory markers: myeloperoxidase, tumor necrosis factor-alpha and interleukin-6, were inhibited in the striatum, prefrontal-cortex, and hippocampus. Also, diosgenin improved the levels of neurotrophic factor in the three brain regions in both protocols respectively. Among other mechanisms, the antipsychotic effect of diosgenin might be associated with attenuation of neurochemical and neuroimmune alterations.

List of abbreviations

SCZ	schizophrenia
5-HT	5-hydroxyltryptamine
GAD	glutamic acid decarboxylase
KET	ketamine

* Corresponding author at: DELSU Joint Canada-Israel Neuroscience and Biopsychiatry Laboratory, Department of Pharmacology, Faculty of Basic Medical Sciences, College of Health Sciences, Delta State University, Abraka, Delta State, Nigeria.
E-mail address: bben-azu@delsu.edu.ng (B. Ben-Azu).

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NMDA	N-methyl-D-aspartate
DG	diosgenin
RIS	risperidone
OFT	open-field test
NORT	novel object recognition test
WBT	wood-block test
FST	force swim test
MPO	myeloperoxidase
TNF-α	tumor necrosis factor-alpha
IL-6	interleukin-6
VTA	ventral tegmental area

Introduction

Schizophrenia (SCZ) is one of the commonest psychotic disorders that affects approximately 24 million people with a global prevalence of about 1% [1]. It is regarded as a serious neuropsychiatric disorder clinically typified by three groups of indicators. These includes the positive symptoms such as delusions, hallucinations, stereotypy; negative symptoms including affective flattening and social isolation [2], as well as core symptoms of learning and memory deficits [2,3]. While principally manifesting at adulthood, SCZ has been reported to have neurodevelopmental origin with connections to genetic factors and environmental assaults [1].

Despite the increased burden of SCZ, the pathophysiology of the disease remains elusive. However, it has been hypothesized to be linked to oxidative stress, neuroinflammation and neurochemical imbalances. These assaults change different genetic makeups, notably altering several biological processes and pathways in specific brain regions [4–11]. Although these remain controversial, more studies have clearly characterized their involvement and role in the pathological mechanisms of the disease than expected [4–9]. The vulnerability-stress-inflammation model has also been established to support the growing evidence of the role of oxidative stress and inflammation in SCZ [9,12,13]. This model suggests that the genetic alteration could serve as a first-hit factor, notably predisposing vulnerable subjects to increased susceptibility of the second-hit factors such as psychotropic agents, stress and infection, which subsequently exacerbate the inflammatory responses later in life [13]. Mounting body of evidence including meta-analyses, have reported high levels of pro-inflammatory mediators in drug-naive and first-episode schizophrenic patients [14,15]. Similar findings were found in severe psychotic patients, including patients without significant inflammation [16], precisely, patients in the acute relapse and stable outpatients [17]. Of note, different animal models have also shown correlations between elevated pro-inflammatory cytokines and severity of psychotic symptoms of SCZ [9–11]. These facts point out the predominance of neuroinflammatory pathway in the development of SCZ and its related pathological consequences. Specifically, this includes enduring loss of cortical pyramidal neurons due to glutamatergic excitotoxicity resulting from N-methyl-D-aspartate (NMDA) receptor low grade functions [9,18–20]. In terms of antipsychotic effects, previous studies have shown series of therapeutic failure and non-clinical effects of first-generation antipsychotic drugs such as haloperidol unlike the second-generation family like risperidone, in the treatment of the disease [9]. In previous preclinical and clinical reports, haloperidol was shown to exacerbate the inflammatory processes involved in the reoccurrence of SCZ symptoms [9,21]. However, many second-generation agents including risperidone have been shown to profoundly reverse SCZ-like symptoms and pathological mechanisms such as inhibition of neuroinflammation, oxidative/nitrergic stress, reduction of cortisol/corticosterone level, and enhancement of neurotrophic support [9,21–23]. Moreover, unlike haloperidol, risperidone has been shown to suppress neuroinflammation-induced depletion of dendritic branching and synapsis of cortical pyramidal neurons, notably susceptible to neuroimmune changes [9].

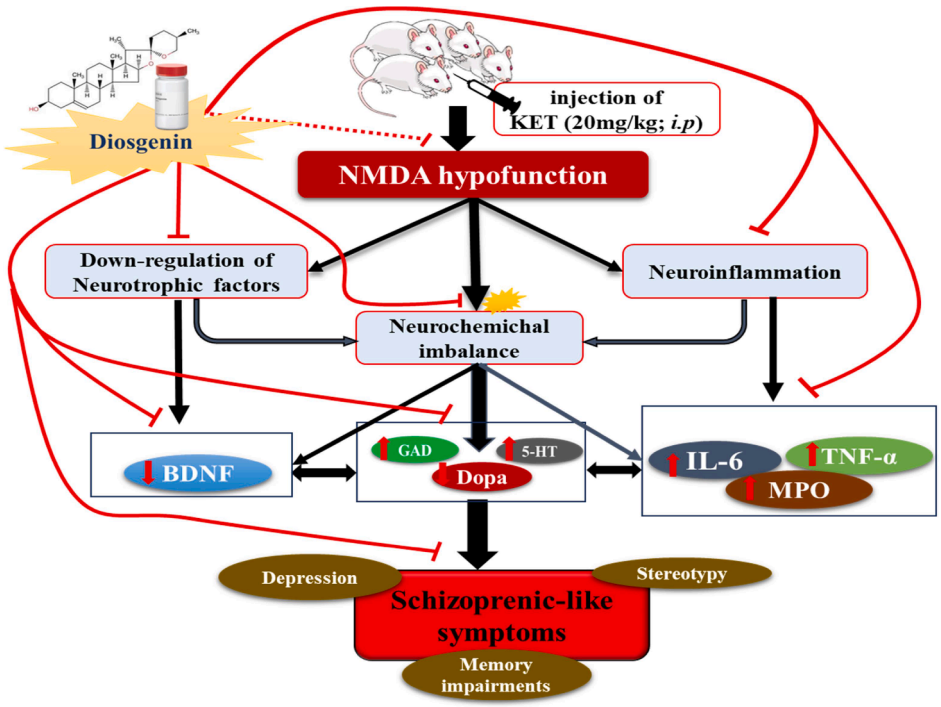
From this perspective, inhibition of anti-inflammatory factors and recruitment of pro-inflammatory mediators generally observed in the early and late (chronic) phases of SCZ are important determining factors to the chronification of psychotic symptoms and therapeutic outcomes of suspected agents with antipsychotic potential [23]. Interestingly, exacerbation of neuroinflammatory processes have been linked to the imbalances observed in the release of neurochemical substances, down-regulation of neurotropic factors/proteins, and neuronal cell death in the different brain regions implicated in the pathogenesis of the disease [7–9,23]. The neurotransmitter theories such as glutamate and dopamine hypotheses remain the core mechanisms explaining pathophysiology of SCZ and some behavioral correlates. High dopaminergic activities leading to exacerbation of mesolimbic dopaminergic signaling concurrently with decreased glutamatergic innervation are presented as main culprits of SCZ [7,24]. However, changes in GABAergic, cholinergic, noradrenergic and 5-hydroxytyptaminergic (5-HTergic) neurotransmissions have also been pin-pointed as other promoters of the psychotic symptoms [7,8]. Moreover, cumulating evidence have shown that the pathophysiology of SCZ was also mediated via impaired cortical dopaminergic and GABAergic neurotransmission as well as neurotrophic changes, primarily owing to glutamatergic excitotoxicity and neuroinflammation [8,25,26]. Based on substantial reports on the core pathogenic mechanisms underlying SCZ and the limitations of current treatments, oxidative burden, neuronal inflammation, and several other pathogenic interplays are increasingly suggested as new therapeutic targets in the treatment of SCZ [9,27,28]. Interestingly, ketamine (KET)-induced SCZ-like phenotypes, characterized of neuroimmune and neurochemical impairments, has progressively improved the understanding and therapeutic outlooks of the underlying patho-mechanisms involved with the disease, especially owing to its ability to induce NMDA receptor hypo-functionality [9,27–30].

Other than the use of antipsychotic drugs in treating SCZ-like symptoms [32], plant-based compounds (nutracuetics) are now considered as treatment and a maintenance option for SCZ and other mental illness due to their availability, low cost-effect [7,27,32–34] and their ability to extenuate SCZ with little or no adverse effects. Diosgenin (DG) is a natural steroidal saponin compound, isolated from several medicinal plants used in the treatment of several pathologies [35]. Considerable reports have shown it antioxidative, anti-inflammatory, neurorestorative, neuroprotective, and anti-apoptotic properties against different neurological disorders [36,37]. In experimental studies, DG has been used to extenuate neurodegenerative diseases like Parkinson's and Alzheimer's diseases associated with cognitive deficits, oxidative burden, and inflammation [36,37]. Recently, our study showed that DG ameliorated alcohol-exacerbated social defeat stress-induced neuroinflammation and neurochemical alterations in mice [38]. Given these beneficial properties shown by DG and incomplete antipsychotic mechanisms of action from our previous study [6], we therefore hypothesized that administration of DG would contain neuroimmune challenge, and ameliorate neurochemical and neurotrophic dysfunctions associated with SCZ (Scheme 1). Hence, this study investigated the neuroprotective and neurorestorative effects of DG in experimental mouse model of KET-induced SCZ and its underlying mechanisms on neuroinflammatory and neurochemical alterations in mice brains.

Materials and method

Drugs and reagents

Diosgenin (DG), risperidone (RIS), pro-inflammatory cytokines and neurochemicals antibodies were purchased from Sigma-Aldrich, St. Louis, MO, USA, Burgoyne Burbidges & Co., Mumbai, India and Elabscience, USA. Ketamine hydrochloride was bought from SWISS Parenteral LTD, Gujarat, India. Other chemicals procured for this experiment were of analytical grades with highest purities.



Scheme 1. Experimental hypothesis.

Experimental animal

Twenty-three to twenty-five gram (23–25 g) male Swiss mice were obtained from the Laboratory Animal Facility of the College of Medicine, Delta State University, Abraka and acclimatized (23±2 °C; 12-hr light/12-hr dark cycle, 40–70 % relative humidity) for two weeks with free access to food and water. The experimental protocol adopted for this study was approved by Delta State University Animal Care and Use Research Ethics Committee of the Faculty of Basic Medical Sciences (REC/FBMS/DELSU/23/185) in concordance with the International Regulatory Agencies such as National institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85–23, revised 1985).

Dose selection and treatment

The doses of KET (20 mg/kg) [7,39], DG (25 and 50 mg/kg) [6,40], and RIS (0.5 mg/kg) [7] were selected in alignment with their previous outcomes. Each of the drugs used was constituted with saline (0.9 %) before oral (*p.o.*) administration. Meanwhile, we diluted KET in 0.9 % saline and then injected intraperitoneally *(i.p.)* to the animals as established from previous protocols [11,39].

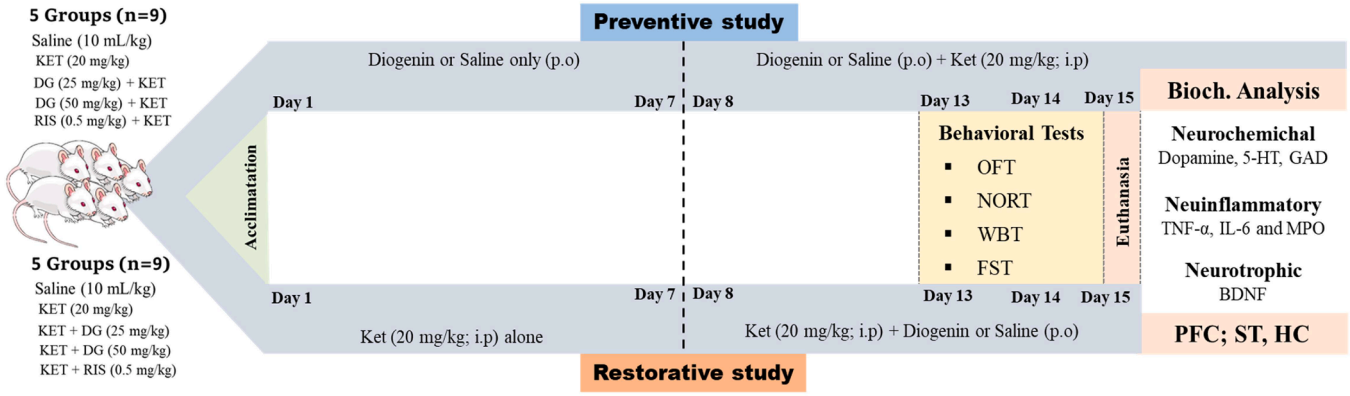
Experimental protocol

After acclimatization, animals were randomly distributed into ten (10) groups of nine (9) mice each and divided into two different experiments to evaluate the preventive and the reversal effects of DG on KET-induced SCZ. Animals in experiment 1 were used to assess the delaying or preventive potency of DG on KET-induced SCZ symptoms while reversal experiment was aimed to evaluate the correcting or reversal properties on established or ongoing SCZ. Accordingly, animals in the preventive experiment were divided into 5 groups ($n = 9$). The animals selected into groups 1 and 2 were administered saline (10 mL/kg, *p.o.*), groups 3 and 4 had DG (25 and 50 mg/kg, *p.o*) and the animals in group 5 received RIS (0.5 mg/kg, *p.o.*) daily for 14 days. However, from days 8–14, the animals in groups 2–5 additionally received daily injection of KET (20 mg/kg, *i.p.*) after oral administration of the vehicle, DG and RIS. For the reversal or restorative study, the animals were also divided into 5 groups ($n = 9$). The animals selected into group 1 were administered saline (10 mL/kg, *p.o.*). Groups 2–5 were injected daily with KET (20 mg/kg, *i.p.*) for 14 days. But from days 8–14, the mice group 2 additionally took saline (10 mL/kg, *p.o.*) and groups 3–5 were treated once per day with DG (25 and 50 mg/kg, *p.o*) and RIS (0.5 mg/kg, *p.o.*) 30 min after KET injection. Behavioral phenotypes of SCZ such as stereotypy, depressive features, and memory deficits were examined in the animals in the two studies. To avoid KET interference effect on the behavioral results, the tests were carried out 24 h after each treatment between days 13 and 15 by behavioral experts blinded to the experiment (Scheme 2).

Behavioral tests

On day 13, stereotypy behavior, which comprises of recurring psychotic behavior, was evaluated in open field-maze (20 cm x 20 cm x 23 cm) for 30 min for each animal as described previously [7,11]. Mice were scored using stereotypy scale of repetitive behavior comprising of 0 = stereotypy, 1 = head movement, 2, intermittent sniffing, 3 = intense chewing, and 4 = licking. Novel object recognition test (NORT) was performed on day 14 to assess non-spatial cognitive dysfunction in the KET-induced SCZ mice as described [34]. Mice are trained with two identical objects (A and B) for 5 min during the trial session to familiarize with both. 24 h later, object B was replaced with a novel object (C) and animals were allowed to investigate objects A and C for another 5 min (test session). The discrimination index (DI) was thereafter calculated as previously described.

To assess the extrapyramidal effects of DG, animals were subjected to the wood-block test (WBT) for cataleptic behavior as previously described [41]. For the assessment of psychotic depressive-like behavior from KET-enhanced immobility on day 15, each animal was investigated for 6 min in a cylinder glass jar with 30 cm in height and 20 cm in diameter [8]. Immobility behavior as observed with movement cessation except floating behavior or light paddling was considered as an index of despair for 5 min after disregarding the first 1 min during which they exasperated to escape.



OFT: Open-Field Test for Stereotypy behavior; **WBT:** Wood-Block Test for Catalepsy

FST: Forced-Swimming Test for Depression; **NORT:** Novel Object Recognition Test for Cognition

Scheme 2. Research design.

Preparation of brain supernatants

Supernatants of the hippocampus, striatum, and prefrontal cortex isolated on a cold-ice tray after euthanasia, homogenization, and centrifugation with cold phosphate buffer (PBS) 10% w/v, 0.1 M, pH 7.4 based on previous protocols [7–11].

Estimation of protein concentration

The protein contents of each brain regions were estimated as previously described. 0.9 mL of distilled water, 0.1 mL of the supernatants and 3 mL biuret reagent were added together and incubated at 25°C for 30 min. The absorbance was read at 540 nm (UV/Vis Spectrophotometer (INESA 750 N, China)). The standard (1 mg/mL bovine serum albumin) was then calculated within 0.01–0.1 mg/mL range [7].

Estimation of myeloperoxidase levels

The level of myeloperoxidase was estimated in the isolated brain regions based on previous protocol [9]. The MPO unit was considered at absorbance change of 0.001/min with the activity was taken as the unit of MPO/mg of protein [9].

Estimation of inflammatory cytokines, neurochemicals and brain derived neurotropic protein levels using enzyme-linked immunosorbent assay

The levels of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), dopamine, and 5-HT were estimated in the isolated brain regions as described by the manufacturer's protocol from ELISA kits, and absorbance were read using the Spectramax M- 5 plate reader (Molecular Devices, Sunnyvale, CA), and calculated from the standard curve of the ELISA kits and then expressed in pg/mg protein. Glutamic acid decarboxylase (GAD) was assayed according to earlier method [11].

Statistical analysis

The data were analyzed using one or two–way analysis variance (ANOVA) and expressed as Mean ± *S*.E.M followed by post–hoc test (Bonferroni) for multiple comparisons where appropriate using Graph Pad Prism software, Inc., Lajolla, USA, version 5.0. A level of $p < 0.05$ was considered as statistically significant for all tests.

Results

Diosgenin prevents and reverses stereotypy in ketamine-induced schizophrenic mice

As indicated in Fig. 1a, b, KET induces significant ($p < 0.05$) increase in stereotypy behavior in the preventive and restorative studies relative to the control groups. In the preventive study alone, treatment with DG (25 and 50 mg/kg, $p < 0.01$) significantly extenuated KET-induced stereotypy behavior relative to the KET control group (Fig. 1a). However, treatment with RIS (0.5 mg/kg) in both study phase significantly ($p < 0.001$) reduced the KET-induced stereotypy behavior relative to the KET control groups (Fig. 1a, b).

Diosgenin enhances non-spatial memory in both protocols in ketamine-induced schizophrenic mice

After non-spatial working memory assessment, it was observed that KET exposure decreased the DI which is considered as an index of non-

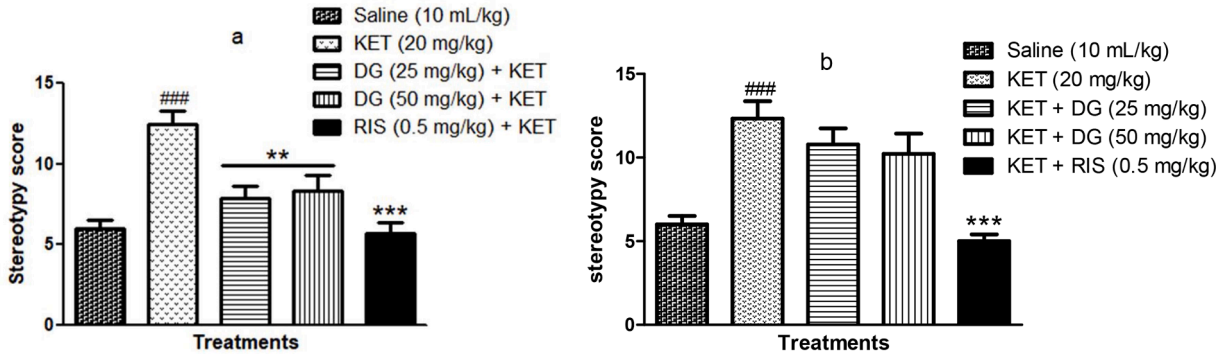


Fig. 1. a, b: Diosgenin prevents (a) and reverses (b) stereotypy in ketamine-induced schizophrenic mice Bars represent the mean ± *S*.E.M of 9 animals / group. ###$p < 0.001$ vs saline group and **$p < 0.01$, ***$p < 0.001$ vs KET group. **KET** = Ketamine, **RIS** = Risperidone.

spatial memory function in animal, in the preventive and restorative studies when compared to the control groups (Fig. 2a, b). Post-hoc analysis indicates that DG (25 and 50 mg/kg) treatment significantly ($p < 0.05$, $p < 0.001$) increased the DI in the preventive and restorative studies comparative to the KET control groups (Fig. 2a, b). Our experiment also showed RIS (0.5 mg/kg) in both studies significantly increased the DI in the preventive ($p < 0.01$) and restorative ($p < 0.05$) protocols comparative to the KET control groups (Fig. 2a, b).

Diosgenin diminishes depressive-like symptoms in ketamine-induced schizophrenic mice

Fig. 3a, b indicates the psychotic depressive-like effect of KET exposure in mice. KET treatment elicited significant ($p < 0.05$) increase in depressive-like behavior as depicted by KET-enhanced immobility time in the preventive and restorative studies comparative to the control groups. We found that DG (25 and 50 mg/kg) significantly ($p < 0.001$) decreased the KET-enhanced psychotic depressive-like behavior in the preventive study comparative to the KET control group (Fig. 3a). While in the restorative study phase, DG (50 mg/kg, $p < 0.001$) alone decreases the KET-induced depressive-like behavior (Fig. 3b). Similar to the DG treatment, RIS (0.5 mg/kg) administration significantly ($p < 0.001$) reduced the KET-induced depressive-like behavior comparative to the KET control groups in both studies (Fig. 3a, b).

Diosgenin lacks cataleptogenic potential in murine experimental mice

For us to check for the potential ability of DG to produce extrapyramidal side effect after prolonged usage, as seen from the preventive treatment of 14 days, we additionally conducted experiment on catalepsy test using the WBT (Fig. 4). The WBT measures cataleptic effect of test compounds based on latency to move away from an enforced position [5]. In this test, there was no difference in the catalepsy time following DG (25 and 50 mg/kg) treatment for 14 days together with KET treatment from days 8–14 comparative to saline and KET controls respectively. Similarly, RIS (0.5 mg/kg) treatment did not indicate any significant differences in catalepsy time relative to the saline and KET control groups (Fig. 4).

Diosgenin modulates the neurochemical substances in ketamine-induced schizophrenic mice

Based on the results in Fig. 5a–f, KET treatment impacted the neurochemicals in the different regions of the mice brain. KET treatment was found to significantly ($p < 0.05$) elevate the release of dopamine in the hippocampus, striatum, and prefrontal cortex regions in the preventive study as well as in the striatal and hippocampal regions in the restorative study when compared to the control groups (Fig. 5a, b). In support of the hypodopaminergic transmission in the cortical area, we found decreased dopamine level in the cortex in the restorative study comparative to the control group (Fig. 5b).

Our intervention with DG (25 and 50 mg/kg) markedly reduced the levels dopamine in the prefrontal cortex and striatum ($p < 0.05$, $p < 0.01$) in the preventive study in comparison with KET control groups (Fig. 5a). In the restorative study, dopamine level in the striatum was reduced ($p < 0.001$) after treatment with DG (25 and 50 mg/kg), while treatment with only 50 mg/kg of DG was found to reduce ($p < 0.05$) the level of dopamine in the hippocampus when compared to the KET control group (Fig. 5b). Interestingly, a significant ($p < 0.001$) up-regulation of dopamine release was found in the cortex after treatment with DG (50 mg/kg) and RIS (0.5 mg/kg) when compared to the KET control group (Fig. 5b). RIS (0.5 mg/kg) treatment reduced significantly ($p < 0.001$) the level of dopamine in the cortico-striatal-hippocampal region in the preventive and restorative studies when compared to the KET control groups (Fig. 5a, b).

As regard the levels of 5-HT in the different brain regions assayed in this study, ANOVA showed that KET treatment was found to significantly ($p < 0.05$) elevate 5-HT levels in the prefrontal cortex and striatum in the preventive study, as well as in the striatum in the restorative study when compared to the control groups (Fig. 5c, d). Post-doc analysis revealed that our intervention with DG (50 mg/kg) and RIS (0.5 mg/kg) significantly decreased the levels 5-HT in the prefrontal cortex and striatum ($p < 0.05$, $p < 0.001$) in the preventive study and in the striatum ($p < 0.05$, $p < 0.01$) in the restorative study when compared to the KET control groups (Fig. 5c, d).

As regards GAD, the synthetic enzyme for conversion of glutamate to GABA, a very important inhibitory transmitter depleted in the disease, we determined GAD level in different brain regions. We found that DG treatment elevated the levels of GAD enzyme in the striatum (50 mg/kg, $p < 0.05$), cortex (25 mg/kg, $p < 0.05$, and 50 mg/kg, $p < 0.01$), and hippocampus (50 mg/kg, $p < 0.01$) in the preventive study relative to the KET control group (Fig. 5e). While in the restorative study, GAD expression was only found to be up-regulated in the striatum after treatment with DG (25 mg/kg, $p < 0.05$ and 50 mg/kg, $p < 0.01$) relative to the KET control groups (Fig. 5f). However, RIS (0.5 mg/kg) treatment elevated the level of GAD expression ($p < 0.05$) in the prefrontal cortex, striatum, and hippocampus in the preventive and restorative studies when compared to the KET control groups (Fig. 5e, f).

Diosgenin abates the increases in neuroinflammatory enzyme and cytokines in ketamine-induced schizophrenic mice

Fig. 6a–f shows the outcomes of DG on MPO and pro-inflammatory cytokine activities in the different regions of the mice brain after

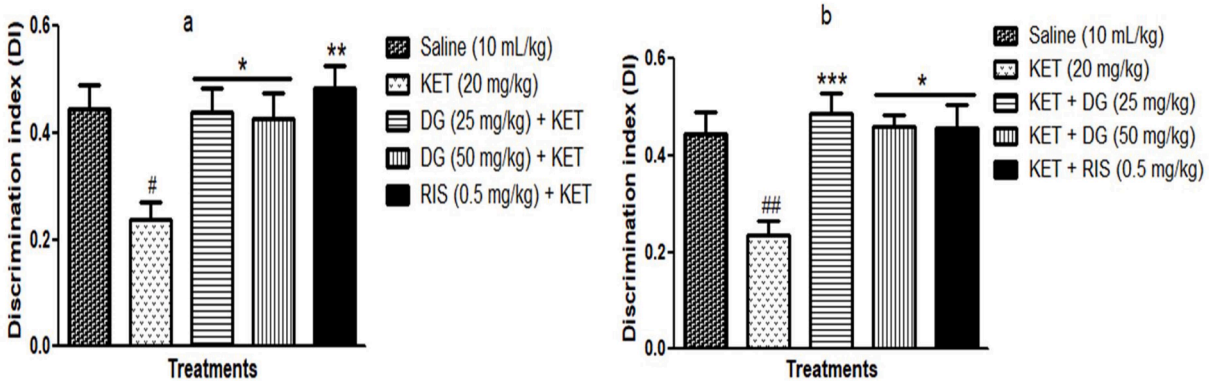


Fig. 2. a, b: Diosgenin enhances non-spatial memory in the preventive (a) and restorative (b) treatment protocols in ketamine-induced schizophrenic mice. Bars represent the mean ± *S*.E.M of 9 animals / group. #$p < 0.05$, ##$p < 0.01$ vs saline group and *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs KET group. **KET** = Ketamine, **RIS** = Risperidone.

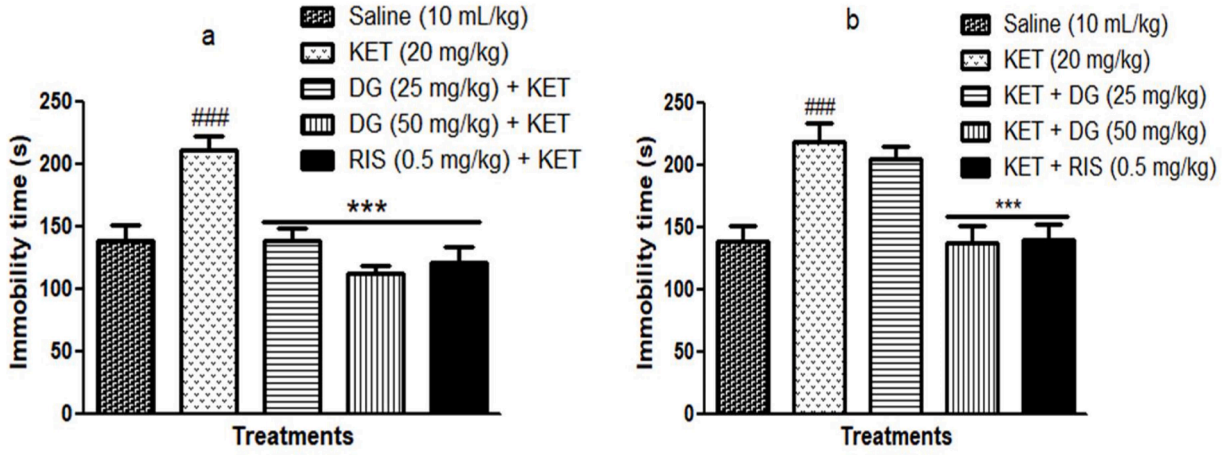


Fig. 3. a, b: Diosgenin diminishes depressive-like symptoms in ketamine-induced schizophrenic mice in the preventive (a) and restorative (b) treatment protocols. Bars represent the mean ± *S*.E.M of 9 animals / group. ###$p < 0.001$ vs saline group and, **$p < 0.01$, ***$p < 0.001$ vs KET group. **KET** = Ketamine, **RIS** = Risperidone.

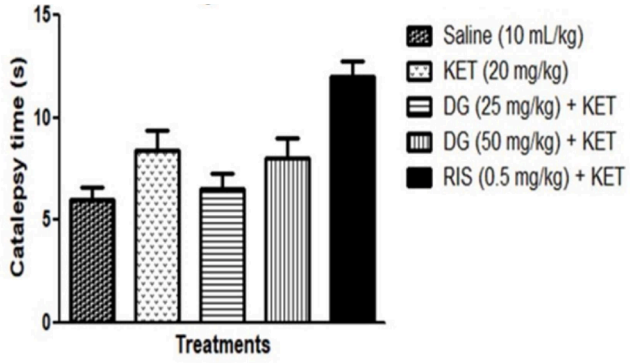


Fig. 4. Diosgenin lacks cataleptogenic potential in murine experimental mice. Bars represent the mean ± *S*.E.M of 9 animals / group. **KET** = Ketamine, **RIS** = Risperidone.

exposure to KET. Post-doc analysis indicates that KET treatment significantly ($p < 0.05$) elevated MPO activity in the three brain regions investigated in the preventive and restorative experiments when compared to the control groups (Fig. 6a, b). DG at 50 mg/kg decreased ($p < 0.05$, $p < 0.01$) the MPO level in the prefrontal cortex and hippocampus, while both 25 and 50 mg/kg of DG were found to decrease ($p < 0.05$, $p < 0.01$) the MPO activity in the hippocampus of the mice brain comparative to KET control group in the preventive study (Fig. 6a). In the restorative study, MPO levels in the prefrontal cortex and hippocampus were reduced ($p < 0.05$, $p < 0.01$) after treatment with DG (50 mg/kg) when compared to the KET control group (Fig. 6b). RIS (0.5 mg/kg) treatment reduced significantly ($p < 0.001$) the level of MPO in the cortical and striatal regions in the preventive, with marked reduction of MPO in the three brain regions in the restorative study when compared to the KET control groups (Fig. 6a, b).

We also found significant ($p < 0.05$) increased TNF-α concentration in the prefrontal cortex, striatum, and hippocampus after KET treatment in both protocols when compared to the control groups (Fig. 6c, d). However, DG (25 and 50 mg/kg) ($p < 0.05$, $p < 0.01$) decreased the level of TNF-α in the cortico-striatal region when compared to the KET controls of the preventive study (Fig. 6c), but in the restorative study, DG differentially reduce the level of TNF-α in the striatum (50 mg/kg, $p < 0.001$), cortex (25 mg/kg and 50 mg/kg; $p < 0.001$), and hippocampus (50 mg/kg, $p < 0.05$) when compared to the KET controls respectively (Fig. 6d). RIS (0.5 mg/kg) treatment reduced significantly ($p < 0.05$) the level of TNF-α in the cortical and striatal regions in the preventive treatment with complementary reduction in the three brain regions investigated in the restorative study when compared to the KET control groups (Fig. 6a, b).

In addition, the IL-6 levels were significantly ($p < 0.05$) elevated after KET treatment in the prefrontal cortex, striatum, and hippocampus in both studies comparative to control groups (Fig. 6e, f). Together, we found that DG (25 and 50 mg/kg) decreased the level of IL-6 in the cortex ($p < 0.01$, $p < 0.05$) and striatum ($p < 0.001$), while 50 mg/kg of DG decreased the level of IL-6 in the hippocampus ($p < 0.05$) when compared to the KET control (Fig. 6e). But in the restorative study, the DG treatment decreased the level of IL-6 in all the regions comparative to KET controls (Fig. 6f). Interestingly, RIS (0.5 mg/kg) significantly ($p < 0.05$) reduced the levels of IL-6 in the prefrontal cortex, striatum, and hippocampus in the preventive and restorative studies when compared to the KET control groups (Fig. 6e, f).

Diosgenin up-regulates brain derived neurotropic factor in ketamine-induced schizophrenic mice

To assay for the neurotrophic support possibly initiated by DG, a neurotropic protein such as BDNF was assayed in the preventive and restorative studies (Fig. 7a, b). Post-doc analysis revealed that DG also prevented KET-induced decreased in BDNF in the striatum (25 mg/kg, $p < 0.05$ and 50 mg/kg, $p < 0.001$) and cortex (50 mg/kg, $p < 0.01$) in the preventive study relative to the KET control groups, in a similar manner to RIS (Fig. 7a). While in the restorative study, the level of BDNF was moreover found to be elevated after treatment with DG in the striatum (50 mg/kg, $p < 0.001$), cortex (50 mg/kg, $p < 0.05$), and hippocampus (25 mg/kg, $p < 0.05$ and 50 mg/kg, $p < 0.01$), relative to the KET control groups (Fig. 7b).

Discussion

In the present study, KET-induce SCZ was characterized with neurobiological sequalae such as neuroimmune changes and neurochemical alterations. Indeed, KET exposure caused an increase in stereotypy behavior, depressive-like phenotype indicative of behavioral despair as well as reduced non-spatial memory functions in the mice. Furthermore, the levels of MPO and TNF-α and IL-6 were significantly exacerbated in the cortico-striatal-hippocampal brain regions of the mice. Apparently, the elevated inflammatory metabolites elicited dysregulated neurochemical release as well as down-regulated BDNF availability in the brain regions investigated relevant to the pathology of the illness. Meanwhile, from our study, we showed that DG tempered the

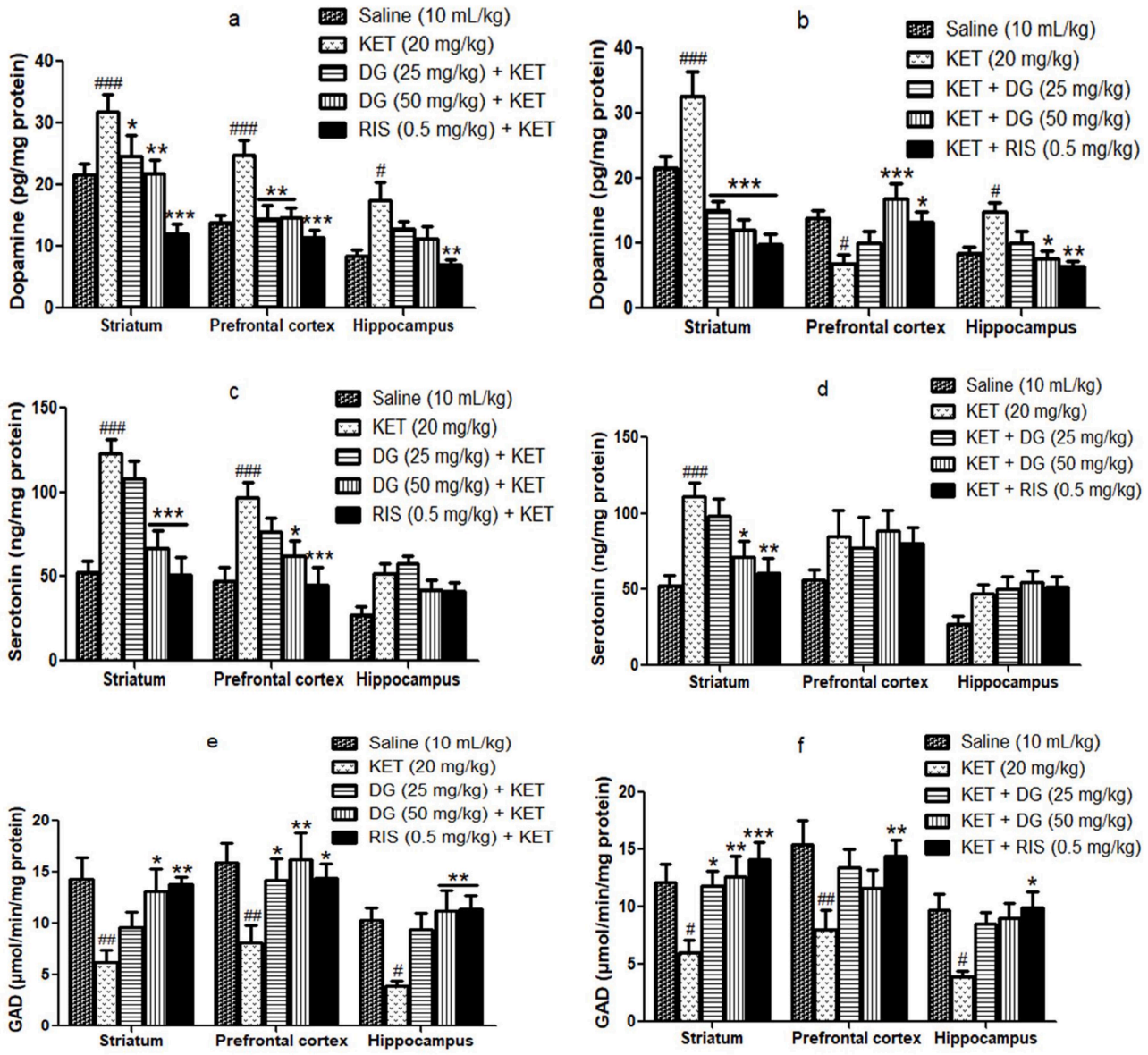


Fig. 5. a–e: Diosgenin prevents (a, c, e) and reverses (b, d, f) ketamine-induced neurochemical dysfunction in mice brains in both protocols. The means ± *S*.E.M each bar are depicted graphically. of 9 animals / group. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs saline group and *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs KET group. **KET** = Ketamine, **RIS** = Risperidone.

neurobehavioral impairments in the mice, notably decreasing stereotypy score and depressive-like behavior, as well as improving the non-spatial memory functions. Our findings also demonstrated that DG treatment, to a very large extent, modulated the immune system by concurrently inhibiting the release of inflammatory mediators and consequently normalizing the dopaminergic, 5-HTergic, and GABAergic system in the prefrontal cortex, striatum, and hippocampus of the mice. More so, DG treatment elevated BDNF in these brain areas of the mice. In this regards, co-administration of DG before or after the NMDA receptor antagonist, KET potentiated the immunoinflammatory and neurochemical system.

Neuropsychological impairments in patients with SCZ and experimental animal models, owing to hypofunctionality of NMDA receptor have been well documented in several research [8,18,19,25,39]. Notably, blockade of the NMDA receptor in the ventral tegmental area (VTA) of the brain have been implicated in the negative and cognitive symptoms owing to its involvement in the control of aversive behavior, reward reinforcement, learning and memory along the striatum and prefrontal cortex [42]. The VTA is an important structure innervated by dopaminergic neurons and in other parts and regulated by glutamate [43]. Besides, GABAergic activity is also connected around the VTA and intricately and significantly depends on dopaminergic release [43]. Previous studies have shown that dysfunction of dopaminergic and GABAergic activities around the VTA was associated with behavioral impairments [44]. Indeed, low levels of dopaminergic activity in the prefrontal cortex, striatum, and hippocampus has been adjudged as one of the underlying factors responsible for the negative symptoms and cognitive impairments [7]. Further, it is evident that blockade of NMDA receptor co-localized in the VTA in human subjects and experimental animals following exposure to KET, elicits psychotic symptoms such as hyperactivity, altered perception with repetitive behavior, impaired verbal fluency, and cognitive deficits [19,44]. From preclinical investigations with KET mouse model of SCZ, mice showed neuropsychiatric despair with significant cognitive decline [11,39], which is consistent with the results obtained in this present study. As earlier reported, psychotic mice showing immoderate behavioral hyperactivity as evidenced by increased stereotypy behavior, was associated with the positive symptoms reported in SCZ patients [5,27]. From our study, we

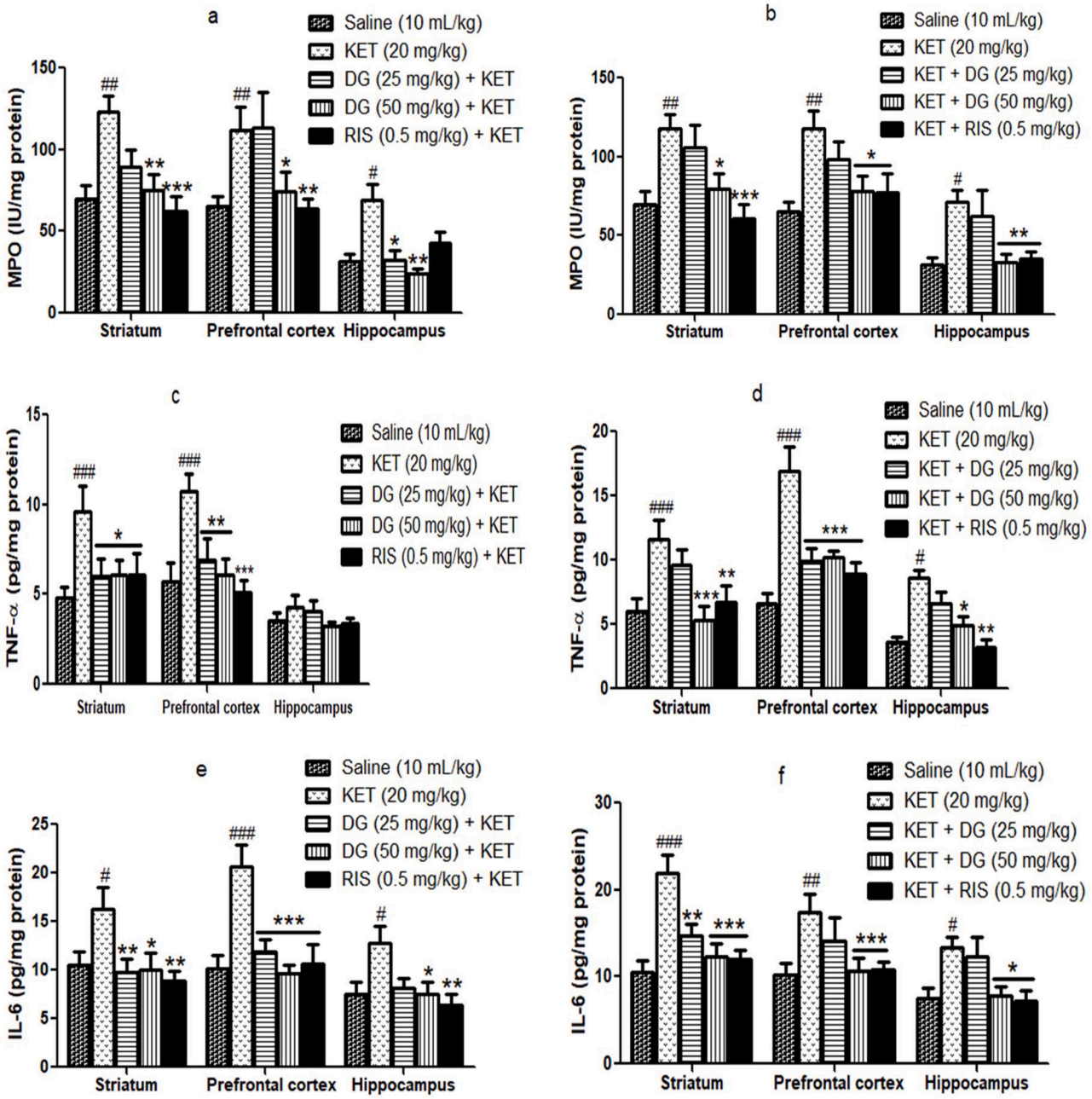


Fig. 6. a–f: Diosgenin abates the increase in neuroinflammatory enzyme and cytokines in ketamine-induced schizophrenia in the preventive (a, c, e) and restorative (b, d, f) treatments. Mean ± *S*.E.M of 9 animals / group are graphically presented. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs saline group and *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs KET group. **KET** = Ketamine, **RIS** = Risperidone.

observed that therapeutic treatment with DG both in the preventive and restorative studies preserved and restored normal behavior by inhibiting psychotic phenotypes including the hyperactivity in the open field arena, depressive-like behavior and cognitive decline of the mice. Perhaps, our findings are similar to the DG's effects in previously related investigations which reported that DG demonstrated conditional anti-depressive-like, anxiolytic and cognitive improvement effects in mice exposed to traumatic stress [36–38,45]. Meanwhile, from the restorative study, treatment with DG did not show significant ameliorative effect in the stereotypy behavior, which might indicate that DG's lack the ability to abate existing positive symptoms of the disease.

The neural circuits accountable for the functional brain networks and cognitive processes have been extensively studied. Many neurobehavioral and cognitive processes are supported by coordinated neural oscillations [46]. Electrophysiological studies have consistently revealed interrupted synchronization of neural circuitry in SCZ patients with first- and severe-episode psychosis linked with cognitive and negative symptoms [47]. However, the synchronization occurs following a finely regulated balance between several groups of inhibitory and excitatory neurons [47]. Notably, GABAergic interneurons control fast firing of pyramidal neurons needed to generate high-frequency rhythms [48]. Accordingly, previous post-mortem investigations of human and animal samples have shown derangements of enzymes and proteins such as GAD and reelin responsible for GABAergic synthesis and transmission, suggesting altered inhibitory system [8,48]. In line with this, alteration of communication between NMDA and glutamate system co-localized with GABAergic neurons was also reported to contribute to the imbalance between excitatory-inhibitory

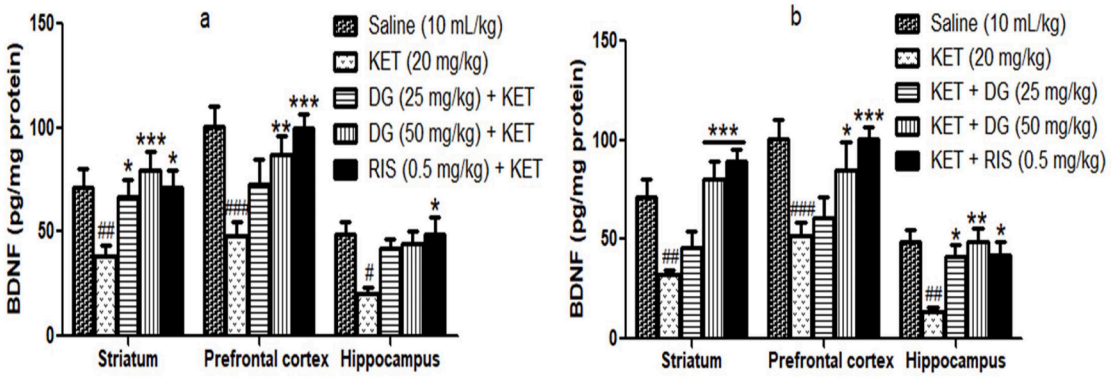


Fig. 7. a, b: Diosgenin up-regulates brain derived neurotropic factor in ketamine-induced schizophrenia in the preventive (a) and restorative (b) treatments. Bars of mean ± *S*.E.M of 9 animals / group are shown graphically. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs saline group and *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs KET group. **KET** = Ketamine, **RIS** = Risperidone.

system [49], as well as deregulation in the release of 5-HT and dopamine [7]. Indeed, up-regulation of 5-HT_{2A} receptor was linked to exacerbation of GABAergic phosphorylation, increased dopamine release, and glutamatergic excitotoxicity [50]. Consequently, these derangements could be linked to behavioral abnormalities resulting from aberrant organization of functional neuronal networks [48,50]. In this study, we observed aberrant elevation of brain dopamine and 5-HT concentration together with reduction in the concentration of GAD in the striatum, prefrontal cortex, and hippocampus, thus affirming the psychotic phenotypes and pathologies induced by KET exposure [5,7,8,11,27]. Previous studies have also shown that KET-induced enduring loss of fast-spike GABAergic interneurons and GAD67 enzyme depletion was linked to up-regulation of enzyme responsible for the synthesis of superoxide radicals [8,51]. Interestingly, these outcomes correlate with the psychiatric phenotypes, notably stereotypy behavior, depression, and cognitive impairments reported in this study. Additionally, the decrease in dopaminergic activity in the prefrontal cortex, also explains the concept of the imbalances in mesoscortical and mesolimbic dopaminergic of SCZ. Specifically, the decline in dopamine in the cortical brain region as shown in the prefrontal cortex has been hypothesized to also contribute to cognitive deficiency, and of note, the negative symptoms reported preclinically and clinically, which in part, might be due to decreased tyrosine hydroxylase and accelerated synaptic uptake of cortical dopamine [52,53]. However, our findings showed DG prevented and restored KET-induced dopamine and 5HT reductions with significant increased GAD concentrations in the striatal, prefrontal cortical, and hippocampal brain regions of the SCZ mice. Importantly, the increase in GAD enzyme, which is an important rate-limiting, decarboxylate enzyme for the synthesis of GABA from glutamate, suggest increased GABAergic neurotransmission [8,11]. Previously, we showed that increase GAD enzyme, dopamine and 5-HT concentrations in the cortical regions significantly contributed to amelioration of negative and cognitive defects associated with the disease [8,11].

The role of immune activation and neuroinflammation in the development of SCZ complications are extensive and include investigations that cover wide range of experimental models, including KET. Measuring potential markers of immune activation and neuroinflammation have been used to study SCZ and clinical outcomes [9,10,20–22]. For example, classical pro-inflammatory cytokines such as IL-2, IL-6 and TNF-α in serum are increased in first episode and acute psychotic relapses in patients diagnosed with SCZ [14,21,22] and experimental model [9,11], but with IL-6 levels found to be reduced following anti-psychotic treatment [9,11]. However, it is important to know that findings have also shown that increase in inflammatory tone occurring at the early phase, preceding the onset of overt psychotic symptoms in SCZ patients is linked to dysregulation of hypothalamic adrenal pituitary (HPA) axis [14,21]. Experimental and clinical data describing dysfunction of HPA axis and its consequences, have shown that HPA axis hyperactivity also plays prominent role in the onset of depressive symptoms and cognitive impairment, notably associated with the negative and cognitive symptoms of SCZ [12,21,34]. Further reports claimed that patients exhibiting core SCZ complications demonstrated complete cardinal features of neuroinflammatory response [20,22]. Among these, marked increase inflammatory enzymes and expression of pro-inflammatory cytokines coupled with abnormal convergence of nuclear factor kappa-B (Nf-kB) genes, high levels of acute-phase reactants, and chemokines activities were found in the blood and cerebrospinal fluids of SCZ patients [22,54]. In line with this, up-regulated level of IL-6 and Nf-kB immuno-expression occurring via a calcium-induced depolarization mechanism, were earlier connected with enduring loss of forebrain GABAergic interneurons such as pyramidal neurons. Consequently, this was notably suggested to be responsible for the unabating depressive and cognitive symptoms of the disease [9,51,55]. Apparently, the measurement of inflammatory cytokines or immune activation may serve as potential markers or predictors of treatment response in the treatment of SCZ [15,20].

In this study, our findings showed that KET induces release of MPO in the prefrontal cortex, striatum, and hippocampus relative to vehicle groups, which was accompanied with increased levels of TNF-α, and IL-6 respectively. MPO is a powerful inflammatory enzyme expressed by neutrophils in response to oxidative stress [9]. It promotes activation of microglia cells, aggravates release of pro-oxidants and pro-inflammatory molecules, and increases permeability and damage of blood–brain barrier [56]. Earlier studies have also shown that heightened brain concentrations of IL-6 during neurodevelopment was also strongly connected with alteration of dopaminergic and 5-HTergic systems [57], suggesting possible role in the onset of neurodevelopmental diseases such as SCZ. In this study, exposure to KET heightened MPO release in the mice cortico-striatal-hippocampal brain region in the preventive and restorative studies which were however attenuated by DG treatment. Since increased brain levels of TNF-α, and IL-6 have been linked to the clinical and molecular features of SCZ [9,51,55], it could be suggested that containment of the levels of pro-inflammatory mediators in SCZ subjects, may serves as a significant clinical marker of antipsychotic agents. Thus, the ability of DG to repress to upsurge of MPO, TNF-α, and IL-6 in a comparable manner to risperidone in the three brain regions investigated in the preventive and restorative studies suggest that inhibition of neuroinflammation might be an important mechanism involved in the anti-psychotic effect of DG.

Furthermore, activation of the neuro-immune system disrupts the release of neurochemicals and synaptic activity in patients with SCZ. Neurotropic protein such as BDNF is an important neurotrophic support factor for synaptic plasticity, neuronal differentiation, and survival [58]. Clinical and preclinical investigations indicate that low serum BDNF

concentration in patients and experimental animals are connected to neurochemical and cognitive impairments [8,23,59]. Consistent with prior investigations [8,25], we also found that low level of BDNF was associated with disrupted dopaminergic, 5-HTergic and GABAergic systems, evidenced by imbalances in neurochemical release in the different brain regions investigated. Here, we showed that the antipsychotic effect of DG was also companied with amelioration of cortical and sub-cortical brain levels of BDNF. This effect, indeed, might be associated with the regulation of dopamine, 5-HT, and GABA in the different brain regions. It is important to mention that owing to the involvement of *BDNF* gene and Val66Met polymorphism in the regulation of cognition, severity of psychotic symptoms, efficacy, and adverse of effects of antipsychotics, modulation of BNDF pathway has become an important therapeutic target for the development of the next group of antipsychotic drugs with minimal side effect, such as extrapyramidal symptoms [60]. Indeed, one of the core clinical challenges linked to prolonged usage of antipsychotic drugs include induction of extrapyramidal side effects, which is due to excessive inhibition of D_2 dopaminergic receptors [5,31]. One therapeutic advantage we can draw from the effect of DG after the 14 days treatment in the preventive study, was the fact that the antipsychotic effect of DG was devoid of extrapyramidal adverse effect, evidenced by cataleptogenic behavior in the mice as shown in the WBT. These findings suggest that the therapeutic effects of DG against psychotic phenotype might, indeed be connected to its ability to normalize BDNF signaling in the striatum, which is one of the popular brain regions known to drive extrapyramidal effect of antipsychotic drugs. We thus, proposed that NMDA receptor modulation with preferential D_2 receptors inhibition, like the second-generation antipsychotic drugs such as risperidone, could also be an important mechanism responsible for this neuroprotective feature of DG. Nevertheless, this assertion requires further investigation prior to substantive conclusion. However, the findings that DG improves BDNF levels in both treatment plans further reinforces its antipsychotic benefits especially against the negative and cognitive symptoms. Notably, this could have also contributed to the absence of the extrapyramidal side effect evidenced by the wood block test. However, further insights are required to determine the brain region dependent effects of DG and the advantages of DG over risperidone as a potential antipsychotic agent for the treatment of SCZ. Although risperidone is well established to improve clinical symptoms of patients with SCZ and protects against depopulation of neurotrophic factors and arborization of cortical pyramidal neurons notably vulnerable to neuroinflammation, oxidative damage, and nitrergic stress [21,31], in the context of tolerability and safety, it is important to reiterate that DG is highly distributed in large quantities in diets with very good safety profile [35]. Moreover, previous elaborate toxicological studies have shown that unlike risperidone which is associated with metabolic adverse effects such as hyperglycaemia, agranulocytosis, kidney failure, seizure, and somnolence [61], DG regardless of routes, lacks toxic effect in humans [62] and rodents [63], as it also serves as an important pharmaceutical industrial agent in the preparation of steroidal and cognitive enhancing drugs [64]. Also, *in vivo* characterization of the effect of DG showed that DG promotes the pharmacokinetic profiles of many drugs, because of it low drug-drug interaction property [65].

In conclusion, the findings from our study showed that DG prevents and reverses KET-induced SCZ-like phenotypes. This effect might be due to mechanisms related to normalization of dopaminergic, 5-HTergic and up-regulation of GABAergic systems owing to inhibition of release of neuroinflammatory mediators such as MPO, TNF-α, and IL-6 in the prefrontal cortex, striatum, and hippocampus of mice brains.

Compliance with ethical standard

All experiments were approved and performed under the guidelines of Faculty of Basic Medical Sciences, Delta State University Animals Ethic Committee (REC/FBMS/DELSU/23/185) and the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication number: 85–23, revised 1985).

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CRediT authorship contribution statement

Benneth Ben-Azu: Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Olusegun G. Adebayo:** Software, Data curation, Conceptualization, Writing – original draft, Writing – review & editing. **Aliance R. Fokoua:** Writing – review & editing, Validation. **Jackson E. Onuelu:** Supervision, Formal analysis, Data curation. **Jerome N. Asiwe:** Writing – review & editing, Software. **Emuesiri G. Moke:** Writing – review & editing, Supervision, Methodology. **Itivere A. Omogbiya:** Writing – original draft, Supervision, Writing – review & editing. **Oghenemarho L. Okpara:** Methodology, Funding acquisition, Data curation. **Jennifer E. Okoro:** Methodology, Funding acquisition, Data curation. **Omadevuaye M. Oghenevwerutevwe:** Resources, Funding acquisition, Data curation. **Christian I. Uruaka:** Writing – review & editing, Resources, Funding acquisition.

Declaration of competing interest

Authors declare that they have no conflict of interest.

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