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1. Neuroscience unit, Department of Physiology, Osun State University, Osogbo, Nigeria;
2. Neuroscience unit, Department of Physiology, Lead City University, Ibadan;
3. Department of Anatomy, Osun State University, Osogbo;
4. Department of Physiology, Adeleke University, Ede;
5. Neuroscience & Inflammation unit, Department of Physiology, University of Ilorin, Ilorin, Nigeria.

*Corresponding author: adedamola.bayo-olugbami@uniosun.edu.ng
ORCID ID: 0000-0002-8967-6503

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ABSTRACT

Neuro-inflammation and oxidative stress have been associated with the behavioral alterations commonly associated with neuropsychiatric disorders including schizophrenia. Bromelain, a phytochemical extracted from pineapple has been recommended as an effective anti-inflammatory, anti-oxidant, antithrombotic, and fibrinolytic agents. However, there is no scientific evidence on its effect on schizophrenia induced by sub-chronic administration of ketamine. Hence the reason for this study. Twenty female Wistar rats randomly grouped into 4, were treated with normal saline 5.0 ml/kg orally; Ketamine 25.0 mg/kg, intraperitoneally (i.p); Bromelain 50.0 mg/kg and Bromelain plus Ketamine (BROM, 50.0 mg/kg + KET, 25.0 mg/kg). Bromelain was administered concurrently and prior to ketamine for 14 days with an interval of 30 minutes. Rats were assessed for behavioral (motor and cognitive) changes. Oxidative, nitrergic, inflammatory markers, and cholinergic (acetylcholinesterase activity) transmission were determined in the cortex or hippocampus. Data were analyzed using one-way ANOVA (Tukey's post hoc), with level of significance set at $p<0.05$. Bromelain prevented hyperlocomotion ($p<0.05$), increased rearing frequency ($p<0.05$) and memory deficits ($p<0.01$) in rats exposed to ketamine compared with ketamine group without intervention. However, there was no significant alteration in grip latency in BROM+KET group vs ketamine. The increased acetylcholinesterase, malondialdehyde and Tumor necrotic factor-α (TNF-α) levels induced by ketamine were reduced by Bromelain only ($p<0.05$) and BROM+KET ($p<0.05$). In addition, rats administered bromelain only ($p<0.001$) and BROM+KET ($p<0.05$) had increased Super oxide dismutase (SOD) level compared with ketamine group. However, prefrontal cortical level of Nitric oxide (NO) was not significantly reduced by BROM+KET compared with ketamine. Conclusively, the neuroprotective effect of bromelain in ketamine induced schizophrenia was associated with modulation of behavior, oxidative stress, neuroinflammation and cholinergic transmission, but not via the nitregic pathway.

Keywords: Bromelain, Schizophrenia, Acetylcholinesterase, Oxidative Stress, Ketamine, Inflammation.
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INTRODUCTION

Schizophrenia is a complex neuropsychiatric disorder that remains a big challenge to neurotherapeutic research and as such has been a major public health concern [1]. Accordingly, its perceptual and behavioral phenotypes are classified into positive (e.g., hallucinations, delusions), negative (e.g., social withdrawal) and cognitive (e.g., executive and working memory deficits) symptoms [2].

Repeated administration of the N-Methyl D-Aspartate (NMDA) receptor antagonist, ketamine, a widely known animal model of schizophrenia has been widely described at least in part, to closely simulate robust groups of positive, negative and cognitive symptoms [2,3]. This is accompanied by approximate neuropathological markers such as oxidative and nitregic imbalances that are often associated with schizophrenia disorder [3,4]. Moreover, ketamine has also been described as an antagonist of alpha-7 acetylcholine nicotinic receptor (α-7nAChR) leading to decreased NMDA-mediated glutamate neurotransmissions via altered nitrergic pathway [3]. Therefore, central oxidative, nitrergic and cholinergic signaling have been hypothesized as possible targets in the pathogenic derangements underlying the pathophysiology of schizophrenia. A large number of antipsychotic agents inhibits or otherwise modulates neurotransmitter systems through multiple mechanisms such as anti-oxidant, anti-acetylcholinesterase, anti-inflammation, immunomodulation among others to ameliorate these symptoms [5,6]. However, evidence from current psychiatric therapy on the efficacy of antipsychotic drugs, particularly of typical antipsychotics on these symptoms remains equivocal due to lack of neuroprotective property [6,7]. Moreover, they have failed to repair the underlying causes of the disease including oxidative, nitrergic and cholinergic alterations but only provide brief relief that is often associated with serious adverse effects including extrapyramidal and metabolic alterations [7,8]. In line with these limitations, there is therefore a critical need for the development of neuroprotective plant based-antipsychotic agents from natural origin capable of targeting the underlying pathological substrates and prevent further neurodegeneration. As such, adopting nutritional pharmacotherapy as a treatment option for schizophrenia condition shows that dietary measure involving nutraceuticals products remain a cost-effective and a naturally viable approach to prevent and extenuate schizophrenia and its associated pathologies [9,10].

Bromelain is a cysteine proteolytic enzyme majorly derived from the stem of pineapple. Its anti-inflammatory, anti-carcinogenic, anti-nociceptive, and antioxidant properties have been established [11]. Its neuroprotective effects have been widely reported [12,13]. It has

also been reported to inhibit the activation of TNF- α [14], which causes inflammatory response. Another study provided evidence that showed that bromelain reverses the increase in cytokines and may exert its prophylactic effect by attenuating inflammation-mediated neuronal degeneration in the 6-OHDA rat model of Parkinson's disease [15]. However, there is no report yet on the effect of bromelain on ketamine-induced schizophrenia. Hence, this study investigated the impact of bromelain administration on behavioral deficits observed in female Wistar rats exposed to subchronic ketamine administration.

Furthermore, we also evaluated its effects on ketamine-induced changes in pro-inflammatory (TNF-α), antioxidant (Superoxide dismutase, SOD), Pro-oxidant (Malondialdehyde MDA; Nitric Oxide NO) in the cortex, as well as Acetylcholinesterase level in the hippocampus.

MATERIALS AND METHODS

Drugs/chemicals:

All chemicals used were of analytical standards. Ketamine was a product of Swiss Parenterals Ltd, (Gujarat, India) and Bromelain powder was manufactured by Khan phytochemicals limited (Rai, India).

Experimental Animals:

Twenty (20) female rats (182-197g) were used for the study. The rats were kept in a well-ventilated animal house of the faculty of basic medical sciences, Osun State University. The rats had free access to clean water and food. The rats were housed in standardized plastic cage maintained between 12-hour light and dark cycle. All protocols and treatment procedures were done according to the Institutional Animal Care and Use Committee (IACUC) guidelines, in strict compliance with the National Institutes of Health (NIH) guideline for the care and use of laboratory animals [16].

Experimental protocol:

The rats were divided randomly into 4 groups containing 5 rats each.

Group one (control) received 5.0 ml/kg of normal saline.

Group 2 received 25.0 mg/kg of ketamine.

Group 3 received 50.0 mg/kg of bromelain.

Group 4 received both bromelain (50.0 mg/kg) and ketamine (25.0 mg/kg).

Bromelain and Ketamine were given concurrently wherein bromelain was administered 30 minutes before ketamine for 14 days. On the last day of bromelain or/and Ketamine administration, behavioral assessments using open field [17], grip [18] and novel object recognition tests [19] were carried out. Twenty- four hours after the last treatment regimen, rats were euthanized for biochemical analysis of cortical levels of Superoxide dismutase (SOD), Malondialdehyde (MDA), Nitric Oxide (NO), Tumor necrosis factor- α (TNF-α) and hippocampal level of Acetylcholinesterase (AChE). The TNF-α

activity was assayed using Enzyme Linked Immunosorbent Assay (ELISA) while others were determined using spectrophotometry.

Behavioral assessment:

On the 14th day of bromelain or/and ketamine administration, behavioral assessment with open field maze, grip test and novel object recognition test were performed.

Open field Test:

Open field test is used to assess the exploratory behavior and motor activities of rodents. The activities of each rat in the maze was recorded for a duration of 7 minutes with a video recorder of high resolution which was kept at a safe distance to cover the entire field. The number(s) of the line crossed (horizontal exploration) and frequency of rearing (vertical exploration) were determined. The test field was cleaned with 70% ethanol and the rat liters were removed to reduce olfactory cues after each experiment.

Grip Test:

This was done using the modified method of [20]. A 25-cm long wooden bar was placed horizontally between two wooden columns. Each rat was held by the tail and allowed to grasp the center of the bar with its forepaws only. The time it takes the rat to fall off the bar was recorded.

Each rat had three training sessions before the actual test.

Novel object recognition test (NORT):

This test assesses non-spatial short term/working memory in animals as initially described by [19] but with slight modification by [17]. A 45 × 50 cm opaque box was used. Rats were allowed to explore two identical objects placed 5 cm apart from each other and from the walls of the box for 10 min (Trial 1; T1) (training section). An inter-trial time (resting phase) of about 30 min was observed after which each mouse was returned to the testing field for the second trial (T2). The time allowed for T2 was also 10 min, during which one of the old objects was replaced with a novel object (test session). Exploration was scored when the nose or vibrissae of the rat was about 2 cm from the object, while sitting on the object was excluded. The box arena and objects used were thoroughly wiped with 70% ethanol after each test before introducing the next rat in order to reduce olfactory cues [19].

The time spent on exploring the old object in T1 and the new object in T2 was used to estimate the memory index calculated as [time spent exploring the new object/total time spent exploring both objects] × 100.

Animal sacrifice, sample collection, and preparation of tissues for biochemical assays:

After behavioral evaluations, rats were euthanized by cervical dislocation. Afterward, transcardial perfusion was done using 50.0 ml 0.1 M PBS (pH 7.4). The rat was decapitated and the brain excised for isolation of the cortex

and hippocampus. This was rinsed in PBS and homogenized over ice in 0.1 M cold sodium phosphate buffer (pH 7.4). The homogenate was centrifuged at 4^{o}C for 10 minutes at 10,000 rpm. The supernatant obtained was aliquoted for subsequent biochemical analysis.

Determination of markers of oxidative stress, inflammation and cholinergic transmission:

SOD:

This was determined according to the method of [21].

MDA:

This was determined as described by [22].

NO: Nitric oxide was assayed according to the method described by [23] using Griess reagent system with a few modifications by [24].

AChE activity:

The procedure described by [25] was used to estimate AChE activity in the cortex.

TNF-α:

The level of TNF-α in the cortex was determined using enzyme-linked immunosorbent assay kits (Nanjing Mornmed Medical, Nanjing City, Jiangsu province, China) following the manufacturer's guidelines. The level of TNF-α was extrapolated from the standard curve and expressed as pg/mg protein.

Statistical Analysis:

Graph pad prism version 8.0 was used for all statistical analyses. All data were expressed as Mean ± SEM.

Data were analyzed using one-way ANOVA and Tukey's post hoc for multiple comparison. P value < 0.05 was considered to be statistically significant.

RESULTS

Effects of Bromelain on number of lines crossed in ketamine-induced schizophrenia rat model:

In Fig 1, exploratory activity as depicted by the number of lines crossed by each rat in the open field test was significantly increased in the ketamine group compared with control ($p<0.05$). In contrast, treatment with bromelain ($p<0.01$) and BROM+KET ($p<0.05$) significantly reduced the number of lines crossed in comparison with the group that received ketamine without intervention.

Effects of Bromelain on rearing frequency in ketamine-induced schizophrenia rat model:

In Fig 2, the rearing frequency which measures vertical exploratory or motor activity, was markedly higher in the ketamine group ($p<0.05$) compared with control. Treatment with both bromelain only and BROM+KET ($p<0.05$) significantly reduced the rearing frequency.

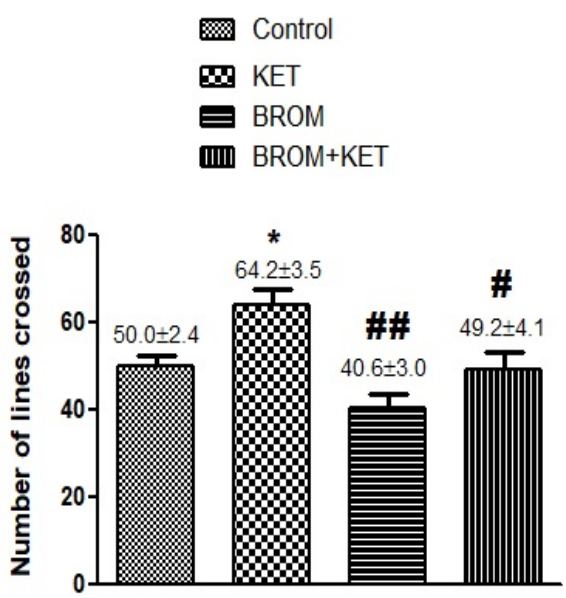

Fig 1: Effects of Bromelain on the number of lines crossed in ketamine-induced schizophrenia like rat model. Values are expressed as mean ± SEM (n=5). *P<0.05 vs control; #P<0.05 vs ketamine; BROM: Bromelain; KET: Ketamine

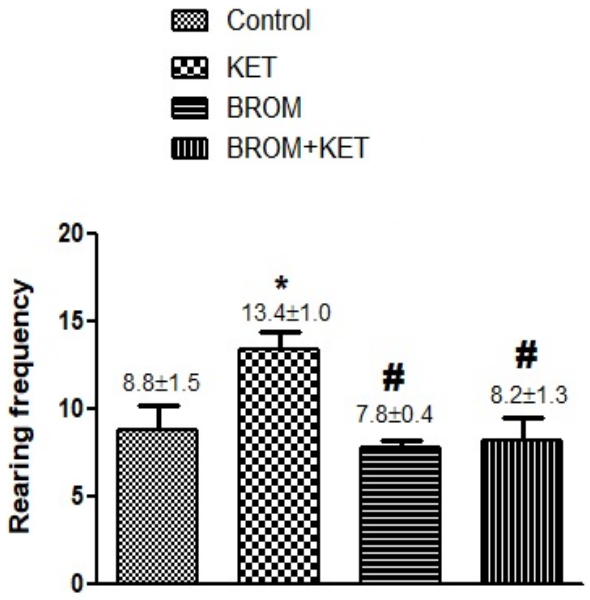

Fig 2: Effects of Bromelain on the rearing frequency in ketamine-induced schizophrenia like rat model. Values are expressed as mean ± SEM (n=5). *P<0.05 vs control; #P<0.05 vs ketamine; BROM: Bromelain; KET: Ketamine

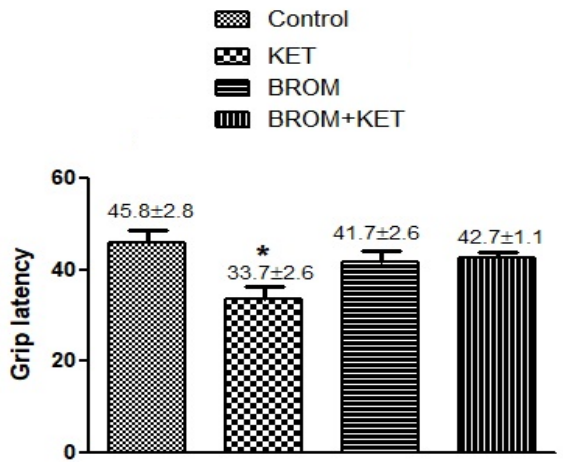

Fig 3: Effects of Bromelain on the grip latency in ketamine-induced schizophrenia like rat model. Values are expressed as mean ± SEM (n=5). *P<0.05 vs control; #P<0.05 vs ketamine; BROM: Bromelain; KET: Ketamine

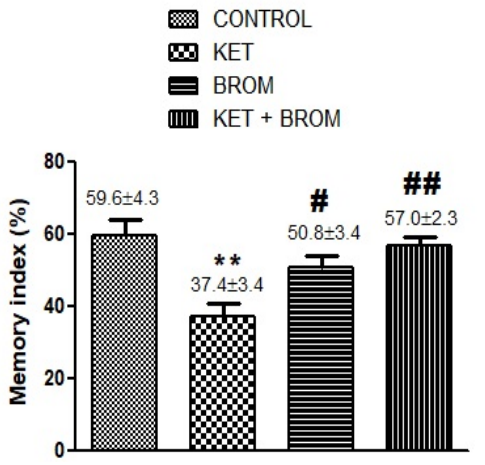

Fig 4: Effects of Bromelain on memory index in ketamine-induced schizophrenia-like rat model. Values are expressed as mean ± SEM (n=5). *P<0.05 vs control; #P<0.05 vs ketamine; BROM: bromelain; KET: ketamine

Effects of Bromelain on grip latency in ketamine-induced schizophrenia rat model

In Fig 3, the grip latency in rats exposed to ketamine ($p<0.05$) without any intervention was markedly reduced compared with control. However, neither bromelain nor BROM+KET could significantly alter the grip latency compared with ketamine group.

Effects of Bromelain on cognitive behavior in ketamine-induced schizophrenia rat model.

As shown in Fig 4, cognitive function as estimated by memory index was markedly reduced in ketamine exposed rat compared with control ($p<0.01$). Administration of bromelain ($p<0.05$) and BROM+KET ($p<0.01$) led to a marked increase in memory index compared with ketamine group.

Table 1: Effects of Bromelain on cortical levels of SOD, MDA, NO, TNF-α & AChE in Ketamine-induced schizophrenia-like rat model.

	CONTROL	KET	BROM	BROM+KET
SOD (u/mg)	7.8±0.6	5.3±0.5*	9.9±0.4##	8.3±0.6#
MDA (uM/g)	0.8±0.1	1.7±0.2**	1.0±0.1#	1.0±0.1#
NO (u/mg)	0.011±0.000	0.014±0.000*	0.011±0.000#	0.012±0.001
TNF-α (pg/ml)	121.5±5.0	158.0±7.0**	127.0±7.5#	125.8±3.0#
AChE (nmol/L)	26.3±2.6	35.1±1.0*	26.3±2.4#	25.2±1.4#

Values are expressed as mean ± SEM (n=5). *P<0.05 vs control; #P<0.05 vs ketamine
BROM: Bromelain; KET: Ketamine; SOD: Superoxide dismutase; MDA: Malondialdehyde; NO: Nitric oxide; TNF-α: Tumor necrotic factor-α; AChE: Acetylcholinesterase.

Effects of Bromelain on markers of oxidative stress, inflammation and cholinergic transmission in ketamine-induced schizophrenia rat model.

As shown in Table 1, there was a significant decline in the level of SOD in the group of rats that received ketamine ($p<0.05$) when compared with control. Rats treated with bromelain ($p<0.001$) or BROM+KET ($p<0.05$) showed significant increase in SOD when compared with ketamine group.

The level of MDA, a marker of lipid peroxidation was significantly elevated in the ketamine exposed rats ($p<0.01$) when compared with control. In contrast, rats that received either bromelain ($p<0.05$) or BROM+KET ($p<0.05$) showed marked decline in the level of MDA in comparison with ketamine group.

NO was markedly elevated in the group that received ketamine ($p<0.05$) when compared with control. However, only bromelain treated rats showed significant reduction in NO when compared with ketamine group ($p<0.05$).

The level of TNF-α was significantly upregulated in the ketamine exposed rats compared with control ($p<0.01$). However, intervention with bromelain or BROM+KET ($p<0.05$) led to a marked reduction in its level when compared with ketamine rats.

There was a significant increase in the level of acetylcholinesterase (AChE), a marker of cholinergic transmission in the group of rats that received ketamine ($p<0.05$) only when compared with control group. However, administration of bromelain ($p<0.05$) and BROM+KET ($p<0.05$) markedly decreased the level of AChE when compared with ketamine group.

DISCUSSION

The present findings show that bromelain treatment reduced ketamine-mediated hyperlocomotion, and memory impairment via regulation of antioxidant system in the experimental rat brains in addition to modulation of inflammation and cholinergic neurotransmission. Motor and cognitive dysregulations which are schizophrenia-associated behavioral impairment have been previously established and reported, as demonstrated by hyperlocomotion and neuropsychiatric phenotypes with poor memory performance using the open field test, novel object recognition test and Y-maze [9,26], which are also similar to the behavioral data obtained in this study. Bromelain is gaining significant attention due to its huge pharmacological potentials, availability and accessibility. Different mechanistic and pre-clinical investigations have been carried out on bromelain to better understand its benefits against various diseases, including disorders of the brain. Interestingly, reviews from different findings proposed that bromelain is a potential and novel multi-target-based pharmacotherapeutic or chemopreventive phytoceutical for the management of variety of chronic diseases such as metabolic and neurological disorders [27]. Importantly, bromelain was reported to show high level of biocompatibility with low toxicity and ease of crossing the BBB. As such, acute and chronic oral administration of bromelain showed no signs of toxicity [28] which therefore, confirms the safety of oral administration of bromelain. Cognitive shutdown and behavioral changes in schizophrenic condition involve a broad array of social cognitive domains [29]. Ketamine injection significantly elicited shift in normal behavioral functions as evidenced with hyperlocomotion (in the OFT) linked to the positive symptoms of psychosis and impairment in spatial memory as evident by reduction in memory index (in the NORT) which is also linked to the negative symptoms of psychosis.

Ketamine, an NMDAR antagonist triggers transient schizophrenia-like behaviors via alteration of the thalamic connectivity when given at sub-anesthetic doses to healthy volunteers [30]. Thus, affirming that NMDAR hypofunction induces the thalamic hyper-connectivity, through the blockage of NMDA

receptors, which affects the GABAergic inhibitory system remarkably involved in the regulation of dopamine and glutamate release in the striatum. This pathological mechanism has been adjudged as one of the major contributory processes that induces and exacerbates severity of some positive symptoms such as hyperlocomotion seen in psychotic patient [30]. Alternatively, reports have also shown that ketamine exposure in experimental psychosis markedly promotes oxido-nitrosative stress and causes neuropathologic release of inflammatory cytokine, which could cause loss of synaptic plasticity in the limbic and cortical brains regions, and consequently affect the cognitive function and neurobehavior of the animals [31].

In this study, preventive intervention with bromelain attenuated psychotic and behavioral deficits phenotypes mediated by ketamine by decreasing hyperlocomotor activity, as well as improving the memory functions, thereby depicting antipsychotic propensity of bromelain in experimental rat. Our results indicated that repeated bromelain administration elicited reduction in hyperactivity in the OFT as depicted by reduced number of line crossings and vertical rearing activity. However, bromelain could not positively alter the grip latency. In similar manner, the spatial recognition memory was also improved as evidenced by increased in time spent exploring the novel object versus old object in the novel object recognition test (NORT). Taken together, these findings could possibly suggest the ability of bromelain to both prevent the positive and negative symptoms of schizophrenia.

It is believed that neurobehavioral shift in psychosis is based on the changes in neurotransmitters activities or release such as acetylcholine imbalance [32]. The severity of schizophrenia depends largely on the level of neurochemical imbalances affecting the neural circuitry [33]. Irregularities in the cholinergic system notably based on acetylcholine (Ach) concentration in the brain has been reported to be responsible for cognitive symptoms of the disorder [34]. Hence, nicotinic agonist and muscarinic antagonist are currently the putative antipsychotic and cognitive enhancing treatment in schizophrenia [30]. More so, the M1 receptor is one of the most abundant cholinergic muscarinic receptors abundantly found in the forebrain and the hippocampus and is a major contributor to the cognitive function in non-schizophrenia brain. However, M1 cholinergic receptor activation leads to the potentiation of excitatory current via the NMDA receptors and contributes to the cognitive functions and neural circuits in schizophrenic patient [35]. In our study, ketamine treatment increased AChE enzyme flux in the hippocampus of rat relative to normal control. As a result, we opined that ketamine might have reduced ACh concentration and subsequently attenuated cholinergic transmission which on the long run, may suppress the nicotinic and M1 receptor. However, intervention with bromelain reverses this effect by reducing AChE enzyme activity,

evidently suggesting increased cholinergic transmission in the hippocampus. Therefore, we attributed the improved motor and cognitive behaviours effected by bromelain to increased cholinergic transmission resulting from reduced AChE enzyme activity. Furthermore, reduced AChE level has been particularly linked to improved neuroprotective functions of cholinergic-anti-inflammatory role of ACh [36]. As such, this present study corroborates the concept that cholinergic system enhancing mechanisms either via AChE enzyme reduction as shown by ketamine or cholinergic receptor agonists from other studies could be responsible for the central neuroprotective function of ACh [37].

To elucidate more on the compelling evidences of redox imbalance in schizophrenia, the involvement of oxidative stress in dysregulating neuronal lipid and altering mitochondrial dynamics causing a shift in neurochemical homeostasis cannot be over-emphasized as it is regarded as one of the culprits underlying the disorder [38]. To corroborate previous investigations [2,39], ketamine injection significantly inhibited the production of SOD enzyme, and increased MDA and nitric oxide concentrations in the prefrontal cortex, suggesting a state of neuronal oxidative stress. Post-mortem investigations of the CSF and cortices of schizophrenic patients show alteration and imbalance in the levels of oxidant-antioxidant enzymes, which contributes to the severity of neuropsychiatric disorders [40,41]. However, enhanced SOD functionality with decreased activity of lipid peroxidation as depicted by reduced MDA level and NO effected by bromelain intervention, suggest its modulatory effect on the oxidant-antioxidant system. High level of reactive oxygen species (ROS) or reactive nitrogen species (RNS) in non-physiological concentration involving cell-signaling activities accompanied by electron transfer reactions favours oxidative stress [42]. Furthermore, oxidative stress reported in this study may further be an inducer of or a product of inflammation. Bromelain treatment demonstrated therapeutically beneficial biochemical effects by enhancing the antioxidant defense system and reducing the brain concentration of MDA by buffering antioxidant arsenals in the brain. TNF-α, an inflammatory cytokine was markedly increased in the ketamine group which may have in part, potentiated oxidative stress. This is usually as a result of excess free radicals generated by cellular metabolic stress and an impaired antioxidant defense system. In contrast, ketamine group treated with bromelain showed decreased level of TNF-α, an observation which is similar to the findings of Adu et al, [43] wherein bromelain reversed increased level of cytokine in an experimental model of Parkinson's disease. This further confirms the anti-inflammatory property of bromelain as earlier posited by [44].

Conclusively, bromelain administration improved schizophrenia-related symptomatic behavior in rats exposed to ketamine, notably by enhancing antioxidant, anti-inflammatory and cholinergic activities. It inhibited lipid peroxidation but did not modulate the nitrergic pathway.

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