***Original Research Article***

**Theaflavin Attenuates Hyperactivity and Cognitive Deficits in a Rodent Model of Prenatal Alcohol-Induced ADHD**

**Abstract**

Attention-deficit/hyperactivity disorder (ADHD) is a prevalent neurodevelopmental disorder characterized by persistent patterns of hyperactivity, inattention, and cognitive impairment. Prenatal alcohol exposure (PAE) has been identified as a significant environmental risk factor for ADHD, leading to structural and functional alterations in brain regions such as the striatum, and amygdala. Current pharmacological interventions for ADHD, while effective, are often associated with adverse effects and limited efficacy in addressing cognitive deficits. Theaflavin, a bioactive polyphenol in black tea, has shown neuroprotective potential. This study evaluates the potential of theaflavin to alleviate ADHD-like symptoms in a mouse model of prenatal alcohol exposure (PAE). Pregnant mice were administered alcohol 20mL/kg (10 mL/kg twice daily) from gestational day GD 7-16 to induce ADHD-like deficits in offspring. Following birth, male pups underwent an initial behavioral assessment on GD 22 to confirm ADHD-like symptoms using Y-maze, the open field test (OFT), and the marble burying test (MBT), after which they were allotted into five groups: control, ethanol-exposed (pathologic group), two theaflavin treatment groups (10 mg/kg and 20 mg/kg), and a positive control group receiving atomoxetine (1 mg/kg). After 30 days of treatment, a second behavioral assessment was conducted to ascertain the effect of the theaflavin. Histological evaluation of the striatum and amygdala was conducted to determine structural changes. Results indicated that theaflavin significantly improved behavioural deficits predictive of ADHD. There was an increase in percentage correct alternation, reduced arm entries as seen in the Y-maze. A significant decrease in duration of ambulation in the open field test, and decreased marble displacement in the marble burying test in a dose-dependent manner compared to the PAE group. Histological analysis revealed improved neuronal density in the striatum and amygdala of theaflavin-treated groups. Theaflavin mitigated neuronal damage exerting it’s neuroprotective effects highlighting its potential as a therapeutic candidate for ADHD management.

**Keywords:** *Attention, theaflavin, hyperactivity, deficit, neurodevelopment.*

1. **INTRODUCTION**

Neurodevelopmental disorders are a group of conditions that begin in early childhood and involve impairments in the development and functioning of the brain and central nervous system (Scattolin et al., 2022; Gogineni et al., 2023). These disorders typically affect a person’s ability to learn, speak, move, behave, or socialize appropriately. They often become noticeable in infancy or early childhood and may persist throughout life. They include autism, attention deficit hyperactivity disorder, epilepsy, and intellectual disorders (Visser et al., 2016; Akpovwre et al., 2024)

Attention-deficit/hyperactivity disorder (ADHD) is one of the prevalent neurodevelopmental disorders affecting children and adults (Song et al., 2021). It is characterized by a persistent pattern of inattention, hyperactivity, and impulsivity that interferes with functioning and development (Frank-Briggs, 2011). These core symptoms can manifest in various ways, including difficulty focusing on tasks, excessive fidgeting, interrupting others, and acting without thinking (Alsaied, (2024). While the etiology of ADHD is complex and multifactorial, genetic and environmental factors are believed to play significant roles (Al‐Gailani et al., 2024; Tistarelli et al., 2020). Prenatal alcohol exposure (PAE) has been implicated as a potential environmental risk factor contributing to the development of ADHD-like symptoms (Popa et al., 2024). Alcohol readily crosses the placental barrier and disrupts critical neurodevelopmental processes, impacting brain structure and function (González-Flores et al., 2024). This disruption can lead to a range of cognitive and behavioral deficits in offspring, including difficulties with attention, impulse control, and motor activity, which closely resemble ADHD symptoms (Aliabad et al., 2024; Montalva-Valenzuela et al., 2022).

Animal studies provide valuable insights into the neurobiological mechanisms underlying the association between PAE and ADHD-like behaviours (Bogdańska-Chomczyk et al., 2024). Researchers use animal models to investigate the specific effects of PAE on brain development and behaviour in a controlled environment (Kim et al., 2024). These studies have consistently demonstrated that PAE results in hyperactivity, attentional deficits, and impulsivity, mirroring the core symptoms of ADHD observed in humans. Andreu-Fernández et al., (2024) observed increased locomotor activity and attentional challenges in rats prenatally exposed to ethanol, providing further evidence for the detrimental impact of alcohol on brain development and supporting the use of this model for studying ADHD-related behaviours.

This research underscores the potential for PAE to serve as a valid model for investigating the neurobiological underpinnings and potential treatment strategies for ADHD and related neurodevelopmental disorders. Understanding the link between PAE and ADHD-like symptoms is crucial for developing prevention strategies and interventions aimed at minimizing the adverse effects of prenatal alcohol exposure on child development.

Theaflavins, complex polyphenolic compounds formed during the fermentation process of black tea, are increasingly recognized for their potential neuroprotective benefits (Akpovwre et al., 2024; Alavi et al., 2025). These compounds are potent antioxidants and possess significant anti-inflammatory capabilities, both of which are critically important in counteracting neuroinflammation (Liu et al., 2024). Neuroinflammation, a hallmark of many neurological disorders, including those related to prenatal alcohol exposure (PAE), involves the activation of immune cells in the brain and the release of inflammatory molecules (Padilla-Valdez et al., 2024; Zeng et al., 2024). Emerging research suggests that theaflavins can play a therapeutic role in mitigating the detrimental effects of neuroinflammation. Specifically, studies in animal models have shown that theaflavins are capable of ameliorating cognitive impairments, such as memory deficits and learning difficulties (Akpovwre et al., 2024), and reducing depressive-like behaviours often associated with conditions like PAE (Wang et al., 2024). This protective effect appears to be mediated, at least in part, by theaflavins' ability to modulate microglial activation (Farkhondeh et al., 2022). Microglia, the resident immune cells of the brain, can become over-activated in inflammatory conditions, exacerbating neuronal damage (Bi et al., 2021). Theaflavins appear to dampen this over-activation, thereby reducing the levels of pro-inflammatory cytokines – signaling molecules that contribute to the inflammatory cascade (Maiti et al., 2022). According to the study conducted by Unno et al. (2019) administration of theaflavins effectively improved memory deficits and significantly reduced inflammation-induced behavioral changes in a mouse model, highlighting the promising potential of these compounds in combating neurological dysfunction linked to inflammation.

Given the behavioural parallels between PAE-induced deficits and ADHD symptoms, investigating the potential of theaflavins to attenuate hyperactivity and cognitive impairments in a rodent model of PAE-induced ADHD is warranted. Thus, this study aims to explore the efficacy of theaflavins in mitigating behavioural abnormalities associated with PAE, thereby contributing to the development of novel therapeutic strategies for ADHD.

1. **MATERIALS AND METHODS**

**2.1 Equipment and apparatus**.

ATKE Centrifuge model C-250 (2023), Equitron Water Bath EWB-30 (2023), Spectrophotometer Inesa 752N Pro (2023), pH meter EDT 3000 (2023), BioTrack Activity Cage (2023), Behavioral Y-Maze Kit (2023), Behavioral open field chamber (2023), weighing balance Ohaus Scout SPX (2023), test tubes Pyrex Test Tubes (2023), Eppendorf tubes Eppendorf Safe-Lock Tubes (2023), tube racks Labcon Tube Rack (2023), plastic cylinders Corning 50-mL Conical Centrifuge Tubes (2023), R. Michael Dissection Kit (2023)and boards.

**2.2 Drugs and Chemicals**

5,5′-dithiol-bis(2-nitro benzoic acid)-DTNB (Aldrich, Germany), trichloroacetic acid-TCA (Burgoyne Burbidges and Co., Mumbai, India), thiobarbituric acid-TBA (Guanghua Chemical Factory Co. Ltd., China), Tris (hydroxymethyl)-amino-methane (Tris-buffer) (Hopkin and Williams Company, USA), Acetic acid (Sigma-Aldrich, Inc., St Louis, USA), NaHCO3 (BDH Chemicals Ltd, Poole, England), Sodium Carbonate (Fisons, Loughborough Leics, England), Na2HPO4.H2O (BDH Chemical Ltd, Poole, England), NaH2PO4.H2O (BDH Chemical Ltd, Poole, England), K2HPO4 (BDH Chemical Ltd, Poole, England), K2Cr2O7 (BDH Chemical Ltd, Poole, England), KCl (BDH Chemical Ltd, Poole, England), NaOH (J.T Baker Chemicals Co., Phillipsburg, N.J., USA), DMSO (J.T Baker Chemicals Co., Phillipsburg, N.J., USA), theaflavin (BDH Chemical Ltd, Poole, England), and alcohol (Sigma-Aldrich, Inc., St Louis, USA), were used in the study.

**2.3 Experimental Animal**

Adult albino Swiss mice was used in this study, and they were obtained from the Animal House of the Faculty of Basic Medical Science and were housed in plastic cages at room temperature. They were fed with balanced rodent pellet diet and water *ad libitum*. Mice were acclimatized before the commencement of experiments. The experimental protocols were submitted for approval to the Research and Ethics Committee of the faculty of Basic Medical Science (RBC/FBMC/DELSU/24/629) and the experiment was performed in accordance with the NIH Guideline for the Care and Use of Laboratory Animals.

**2.4 Drug Preparation and Treatment**

Theaflavin (TF) was prepared by dissolving 350 mg in 35 mL of 1 % dimethyl sulphoxide (DMSO) to obtain the stock solution as previously described by Akpovwre et al., (2024). The stock solution was further diluted with distilled water to obtain the working concentrations used in the study. Absolute alcohol of 25%v/v was then diluted to obtain a volume of 20mL/kg following the protocol adopted by Choi et al., (2012). Animals were divided into 5 groups (GR 1 – GR 5). GR 1 served as control, while GR 2 -GR 5 received alcohol 20mL/kg (10 mL/kg twice daily) from gestational day (GD) 7-16. GR 3 and 4 received theaflavin at 10 mg/kg and 20 mg/kg respectively. GR 5 served as the positive control and received atomoxetine. Neurobehavioral assessments were carried out on postnatal day 22 to confirm the mice that were symptomatic for ADHD (displaying inattention, hyperactivity and impulsivity) before commencement of treatment after which a second behaviuoral assessment was done on postnatal day 57. Mice were sacrificed on postnatal day 58.

**2.5 BEHAVIOURAL TEST**

**2.5.1 Effect of TF on cognition and hyperactivity using the Y-maze**

Memory function altercation was evaluated using the Y-maze spatial working memory task as

previously described by Omogbiya et al. (2021) with little modifications. Briefly, The Y-maze

apparatus is composed of three equally spaced arm chambers (120°, 41 cm long, 15 cm high and

5cm wide) labeled alphabetically, A, B, and C. Each mouse was made to undergo an exploration test. In which, it was placed into arm A of the apparatus and allowed to move freely. That is, explore the three chambers for 5 min without reinforcement. An arm entry was scored when the four paws of the animals were completely in the arm of the Y-maze, while alternation was measured when an animal entered into all three arms in consecutive order. The following parameters were determined or measured:

• Frequency of arm entries by animals as an indices of hyperactivity

• Alternations (consecutive navigations across all three arms i.e., ABC, CAB, or BCA but not

BAB or ABA). At the end of the experiment, the percentage correct alternation (an index of spatial working memory) was calculated using the formula:

**2.5.2 Effect of TF on hyperactivity using the open field test (OFT)**

The effect of TF on hyperactivity was assessed using the open field test apparatus, which was constructed using gray plastic wood as the material, following the measurements described earlier (Näslund, 2021). A light source of a 35 W bulb was suspended approximately 1 m above the apparatus for background lighting. Mice were carried to the testing room in their home cages, handled by the tails, and placed in the apparatus and allowed to explore the field for 20 minutes. After each trial, the apparatus was cleaned using 70% alcohol and allowed to dry. The exploration was then recorded for later analysis and the center zone and periphery zone were defined, where the center zone is 34.5 cm × 34.5 cm and duration of ambulation was obtained in seconds.

**2.5.3 Effect of TF on impulsivity using the Marble Burying Test**

The Marble Burying Test (MBT) is a widely used behavioural assay primarily utilized to evaluate impulsive, repetitive, anxiety, and compulsive-like behaviors in rodents, particularly mice (Thomas et al.,2009; Schneider and Popik, 2007). The test is based on the natural tendency of rodents to dig and bury objects in their environment. A standard testing cage (typically a transparent plastic cage) is filled with 5 cm deep bedding material, such as wood shavings or corncob. A set number of glass marbles (usually 20) are evenly spaced on the surface of the bedding. The marbles are typically uniform in size (about 1.5 cm in diameter). Before the test, mice are usually acclimated to the testing room to reduce novelty-induced anxiety. Each mouse is placed individually into a cage containing the marbles and allowed to explore for a specified period, typically 30 minutes. After the testing period, the number of marbles displaced is recorded. Increased marble displacement is often interpreted as a sign of increased anxiety or compulsive behavior, whereas decreased displacement may indicate reduced anxiety or compulsiveness, often used in pharmacological studies to evaluate the effects of anxiolytic or anti-compulsive agents (De Brouwer et al., 2019).

## **2.6 HISTOLOGY AND ESTIMATION OF NEURONAL DENSITY**

After the behavioural tests, mice (n = 3) in the respective groups were euthanized with ether. Thereafter, the mice were perfused and their brains harvested and fixed with 10% phosphate buffered formaldehyde used for the procedure. Representative brain tissue sections of each treatment group were stained with Hematoxylin and Eosin to demonstrate general histology of the striatum and amygdala according to the method of Eltony and Elgayar, (2014). Thereafter, images were acquired using an Optronics Digital Camera connected to a computer interface (MagnaFire) and an Olympus BX-51 Binocular research microscope. The general structure of the pyramidal cell, peri-glomerular and granule cells were characterized using inter-reader variability. Viable neuronal cells were counted using Image J at X400 or X250 at different microscopic fields for all groups using the method described by Going, (1994). Sanya Viable neuronal cells were defined as round-shaped, cytoplasmic membrane-intact cells, without any nuclear condensation or distorted aspect. Neuronal density was calculated as a ratio of viable neuronal cells to square area of the circular view in a section.

## **2.7 STATISTICAL ANALYSIS**

All data were presented as Mean ±SEM. The results was analyzed by one-way analysis of variance (ANOVA), and post hoc tests (Tukey test) was carried out to determine the source of significant main effect using Graph Pad InStat® Biostatistics software. The level of significance for all tests was set at α0.05.

1. **RESULTS**

**3.1 The effect of theaflavin on cognition, inattentiveness and hyperactivity using the Y-maze**

**in mice model of ADHD induced prenatally by alcohol.**

The effect of Theaflavin (TF) administered daily for 30 days on cognitive behaviour, attention span and hyperactivity, as measured by the number of percentage correct alternation in the Y-maze test, is shown in **Figure 1 and Figure 2.**

One-way ANOVA from figure 1 showed that there was significant differences between treatment groups. Post hoc analysis using Tukey’s test revealed that TF (10, 20 mg/kg) given daily for 30 days produced a significant (p<0.05) improvement in attention span, as evidenced by an increased percentage of correct alternation when compared with the alcohol group only. TF at 20 mg/kg showed the most significant (p<0.05) improvement compared to the alcohol group only, while the alcohol group showed a significant (p<0.05) reduction in attention span as depicted by a reduced percentage correct alternation when compared to the control group.

One-way ANOVA from figure 2 shows that there was significant differences between treatment groups. Post hoc analysis using Tukey’s test revealed that TF (10, 20 mg/kg) given daily for 30 days produced a significant (p<0.05) decrease in hyperactivity, as evidenced by the reduced the number of arm entries in the Y-maze when compared to the alcohol group only, while the alcohol group showed a significant (p<0.05) increased hyperactivity as seen in the increased number of arm entries when compared with the control.



**Figure 1: The effect of theaflavin on cognitive behaviour and attention span in mice model of ADHD induced prenatally by alcohol using the Y-maze.**

Values represent the mean ± S.E.M for 5 animals per group

# p<0.05 compared to the control group (ANOVA followed by Tukey’s post hoc test).

\* p<0.05 compared to the pathologic group (ANOVA followed by Tukey’s post hoc test).



**Figure 2: The effect of theaflavin on hyperactivity in mice model of ADHD induced prenatally by alcohol using the Y-maze.**

Values represent the mean ± S.E.M for 5 animals per group

# p<0.05 compared to the control group (ANOVA followed by Tukey’s post hoc test).

\* p<0.05 compared to the pathologic group (ANOVA followed by Tukey’s post hoc test).

**3.2 The effect of theaflavin on hyperactivity using the Open field test in mice model of ADHD induced prenatally by alcohol.**

The effect of Theaflavin (TF) administered daily for 30 days on hyperactivity, as measured by the duration of ambulation as shown in **Figure 3.**

One-way ANOVA showed that there was significant differences between treatment groups. Post hoc analysis using Tukey’s test revealed that TF (10, 20 mg/kg) given daily for 30 days produced a significant (p<0.05) reduction in hyperactivity, as evidenced by a decreased duration of ambulation when compared with the alcohol group only. While the alcohol group showed a significant (p<0.05) increase in hyperactivity as evidenced by a increase in the duration of ambulation when compared to the control group.



**Figure 3: The effect of theaflavin on hyperactivity in mice model of ADHD induced prenatally by alcohol using the Open field test.**

Values represent the mean ± S.E.M for 5 animals per group

# p<0.05 compared to the control group (ANOVA followed by Tukey’s post hoc test).

\* p<0.05 compared to the pathologic group (ANOVA followed by Tukey’s post hoc test).

**3.3 The effect of theaflavin on Impulsivity in mice model of ADHD induced prenatally by alcohol.**

The effect of Theaflavin (TF) given daily for 30 days on impulsivity, as measured by the number of marbles displaced as shown in **Figure 4.**  One-way ANOVA showed that there was significant differences between treatment groups. Post hoc analysis using Tukey’s post hoc test revealed that TF (10, 20 mg/kg) given daily for 30 days produced a significant (p<0.05) decrease in impulsivity as evidenced by a decrease in the number of marbles displaced as compared to the alcohol group only, while the alcohol group produced a significant (p<0.05) increased in impulsivity as evidenced by an increase in the number of marbles displaced as compared to the control group.



**Figure 4: The effect of theaflavin on impulsivity in mice model of ADHD induced prenatally by alcohol using the marble burying test (MBT).**

Values represent the mean ± S.E.M for 5 animals per group

# p<0.05 compared to the control group (ANOVA followed by Tukey’s post hoc test).

\* p<0.05 compared to the pathologic group (ANOVA followed by Tukey’s post hoc test).

**3.4 Photomicrograph of the effect of theaflavin on neuronal density count of the striatum in mice model of ADHD induced prenatally by alcohol.**

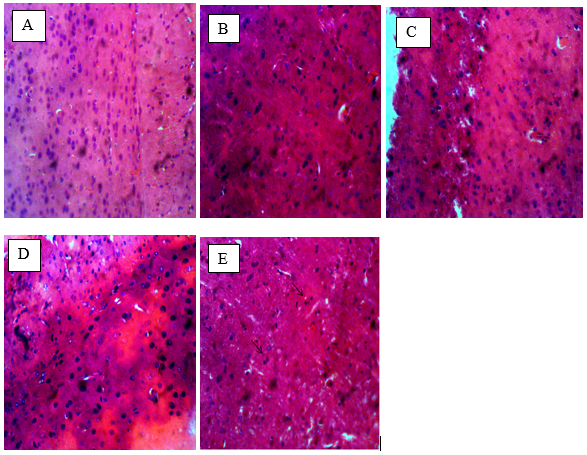


Figure 5a: Representative photomicrograph (HandE-stained section) of the effect of theaflavin on the striatum in mice model of ADHD induced prenatally by alcohol.

Magnification = HE × 400. A= Control, B= ALC, C= ALC+ TF 10mg/kg, D= ALC+ TF 20mg/kg, and E= ALC+ ATX 1mg/kg.

Slide A revealed that there is no observable lesion, slide B reveals that there is no observable lesion, slide C reveals no observable lesion, slide D reveals no observable lesion, and slide E reveals there is atrophy of neurons.



**Figure 5b: The effect of theaflavin on the histology and neuronal density count of the striatum in mice model of ADHD induced prenatally by alcohol.**

Values represent the mean ± S.E.M for 5 animals per group

**3.5 Photomicrograph of the effect of theaflavin on neuronal density count of the amygdala in mice model of ADHD induced prenatally by alcohol**

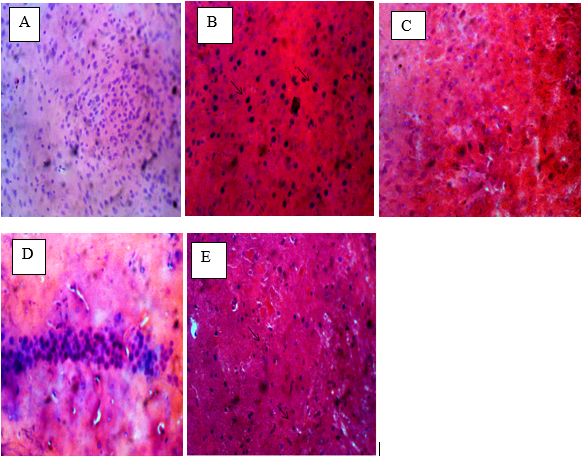


Figure 6a: Representative photomicrograph (HandE-stained section) of the effect of theaflavin on the amygdala in mice model of ADHD induced prenatally by alcohol.

Magnification = HE × 400. A= Control, B= ALC, C= ALC+ TF 10mg/kg, D= ALC+ TF 20mg/kg, and E= ALC+ ATX 1mg/kg.

Slide A revealed that there is no observable lesion, slide B reveals that There is pyknosis of neurons, slide C reveals no observable lesion, slide D reveals no observable lesion, and slide E reveals there is pyknosis of neurons.



**Figure 6b: The effect of theaflavin on the histology and neuronal density count of the amygdala in mice model of ADHD induced prenatally by alcohol.**

Values represent the mean ± S.E.M for 5 animals per group

# p<0.05 compared to the control group (ANOVA followed by Tukey’s post hoc test).

\* p<0.05 compared to the pathologic group (ANOVA followed by Tukey’s post hoc test).

1. **DISCUSSIONS**

This study investigated the therapeutic potential of Theaflavin (TF), a polyphenolic compound derived from black tea (Akpovwre et al., 2024), in ameliorating behavioral and neuroanatomical deficits associated with prenatal alcohol exposure (PAE) in a murine model of Attention-Deficit/Hyperactivity Disorder (ADHD). The findings indicate that TF administration mitigates cognitive impairments, hyperactivity, impulsivity, and neuronal damage induced by PAE.

The Y-maze test primarily used to study memory or cognitive performance in animals (Eduviere et al., 2017; Umukoro and Eduviere, 2016) revealed that TF significantly improved spatial working memory and attention in PAE-exposed mice, as evidenced by increased percentage of correct alternations. These results align with previous research demonstrating the neuroprotective effects of polyphenolic compounds in enhancing cognitive functions by modulating neurotransmitter systems and reducing oxidative stress (Eduviere et al., 2021; Olayinka et al., 2022; Olayinka et al., 2022; **Unno et al., 2019).** The improvements observed suggest that TF may enhance synaptic plasticity and neurotransmission, thereby counteracting the attentional deficits induced by PAE.

In both the Y-maze and Open Field Test (OFT), TF administration resulted in a significant reduction in hyperactivity, as indicated by decreased arm entries and reduced duration of ambulation, respectively. These behavioral changes may be attributed to TF's potential to modulate dopaminergic pathways, which are often dysregulated in ADHD (Cho et al., 2014; Fan and Hess, 2017; Natsheh and Shiflett, 2018; Onuelu et al., 2025). By restoring dopaminergic balance, TF could alleviate hyperactive behaviours associated with PAE.

The Marble Burying Test (MBT) is primarily used in obsessive compulsive behaviour and also used in measuring impulsivity (De Brouwer et al., 2019; Thomas et al., 2009). From the present study, TF significantly decreased impulsivity in PAE-exposed mice, as shown by a reduced number of marbles displaced. This finding suggests that TF may exert anxiolytic effects and improve behavioral inhibition, possibly through the modulation of GABAergic neurotransmission as reported by Egashira et al., (2013) and Taylor, (2017) Such modulation could enhance impulse control and reduce compulsive behaviours.

Histological analyses revealed that TF preserved neuronal density in the striatum and amygdala of PAE-exposed mice. The striatum is critical for motor control and cognitive functions (Cataldi et al., 2022), while the amygdala is involved in emotional regulation (Berboth and Morawetz, 2021). Preservation of neuronal integrity in these regions suggests that TF may exert neuroprotective effects by reducing oxidative stress and inflammation (Anandhan et al., 2013; Anthony et al., 2025; Olayinka et al., 2022) , which are known consequences of PAE. Ahmad et al., (2023) also reported that black tea extract, rich in theaflavins, improved cognitive deficits and reduced acetylcholinesterase activity in Alzheimer's disease models, further supporting TF's neuroprotective potential. These neuroprotective properties of TF could underline the observed behavioral improvements.

1. **CONCLUSION**

Theaflavin exhibits significant therapeutic potential in mitigating behavioural and neuroanatomical deficits associated with prenatal alcohol-induced ADHD in mice. By enhancing cognitive function, reducing hyperactivity and impulsivity, and preserving neuronal integrity in key brain regions, TF may serve as a promising candidate for the development of novel interventions targeting ADHD resulting from prenatal alcohol exposure.

**DISCLAIMER (ARTIFICIAL INTELLIGENCE)**

Author(s) hereby declare that no generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during writing or editing of this manuscript.

**ETHICAL APPROVAL**

The approval for the study was Faculty of Basic Medical Sciences ethical committee (RBC/FBMC/DELSU/24/629) and also in accordance with the NIH Guideline for the care and use of laboratory animals.

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