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Assessment of *Mussaenda pubescens* methanol leaf extract in pain relief, diarrhea, depression, and anxiety in the Swiss albino mouse model

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ABSTRACT

Mussaenda pubescens (*M. pubescens*), a medicinal plant with a long history in traditional medicine, has attracted attention for its potential therapeutic properties. This research intends to assess the *in vivo* pharmacological potentials of its methanolic extract, focusing on the central nervous system (CNS), anxiety, pain relief, and antidiarrheal activity. Phytochemical study revealed the presence of tannins, alkaloids, and flavonoids, which are known for their therapeutic properties. *In vivo*, the analgesic efficacy of the extract was assessed utilizing the abdominal constriction and tail flick methods and displayed potent antidiarrheal effects at the doses of 200 and 400 mg/kg in a diarrheal model induced by castor oil. Additionally, CNS depressant effects were observed in the Open Field Test (OFT) and Hole Cross Test (HCT), while anxiolytic potential was confirmed through the Hole Board Apparatus (HBT) and Elevated Plus Maze (EPM). The anxiolytic effects were found to be dose-dependent, with significant results. These outcomes highlight the significance of *M. pubescens* as a source of bioactive compounds with analgesic, antidiarrheal, and anxiolytic properties. However, additional research is necessary to isolate the active compounds and elucidate the mechanisms of action.

INTRODUCTION

Traditional medicine, particularly the therapeutic application of medicinal plants, continues to hold a pivotal role in global healthcare systems [1]. Despite significant progress in modern medicine, herbal therapies continue to be widely used because of their affordability, easy availability, and perceived lower risk of side effects. Bioactive phytochemicals, endowed with significant pharmacological potential, have been employed for centuries in the treatment of various pathological conditions, and their global utilization is witnessing a resurgence [2].

The World Health Organization (WHO) estimates that around 20,000 plant species possess therapeutic qualities. Among the major health burdens in developing nations, diarrheal diseases persist as a leading cause of pediatric morbidity and mortality [3]. In 2021, diarrheal illnesses accounted for nearly 9% of all deaths in children under five years old, translating to over 1,200 fatalities per day, or an estimated annual toll of 444,000 deaths. Additionally, chronic diarrhea exacerbates malnutrition, further compromising pediatric health. The antidiarrheal potential of traditional medicine has been extensively explored, with nearly 80% of the global population endorsing the efficacy of plant-derived compounds in managing such ailments [4].

Pain, an intricate physiological response to tissue injury, serves as a protective mechanism for the body [5]. Nonsteroidal anti-inflammatory drugs (NSAIDs) mitigate pain and inflammation by inhibiting cyclooxygenase (COX-1 and COX-2), thereby suppressing prostaglandin synthesis. Although prostaglandins act as critical mediators in gastrointestinal mucosal defense, inflammation, and tissue repair, NSAID-induced suppression of their biosynthesis compromises mucosal integrity, predisposing individuals to ulceration and delayed healing [6]. Prolonged NSAID usage is associated with severe adverse effects, including gastric ulceration, tolerance, and dependence. Opioid analgesics, although potent, present challenges related to tolerance and addiction. Consequently, the search for novel analgesic agents with minimal adverse effects remains a global priority [7].

Depression, a prevalent neuropsychiatric disorder affecting up to 20% of the global population, significantly diminishes quality of life and contributes to increased morbidity across all age groups [8]. Conventional pharmacotherapies for mental health disorders such as anxiety, schizophrenia, depression, epilepsy, and Parkinson's disease exhibit limitations, including suboptimal efficacy, undesirable side effects, and potential interactions with other pharmacological agents or dietary components [9]. Consequently, natural compounds have gained traction as complementary or alternative therapeutics for neuropsychiatric conditions [10].

Given the economic feasibility, reduced adverse effects, and global reliance on botanical medicine, estimated by WHO to encompass nearly 80% of the world's population, scientific scrutiny of plant-derived pharmacological agents has garnered significant attention. Medicinal plants serve as an indispensable reservoir for drug discovery, yielding structurally diverse bioactive molecules with profound therapeutic potential [1].

Mussaenda pubescens, a member of the Rubiaceae family, has been traditionally employed for its medicinal properties, with indigenous communities utilizing various plant parts to manage fever, wounds, inflammation, gastrointestinal disorders, and neurological conditions. Despite its extensive ethnomedicinal applications, rigorous scientific validation of its pharmacological effects remains limited, necessitating comprehensive investigations. The exploration of plant-based therapeutic agents is imperative, particularly considering the adverse effects and dependency risks associated with synthetic drugs. Natural alternatives, such as *M. pubescens*, hold promise to address these challenges [11]. *In vitro* studies have shown that *M. pubescens* contains bioactive compounds with notable antioxidant, anti-osteoclastogenic, and detoxifying properties. Its saponins inhibit bone-resorbing cell formation, while extracts reduce toxin absorption in intestinal models and demonstrate strong antioxidant potential due to rich phytochemical content [12-14].

This study aims to elucidate the analgesic, anxiolytic, CNS depressant, and antidiarrheal activities of *M. pubescens*, thereby providing empirical validation for its traditional therapeutic applications. By systematically evaluating its pharmacological potential, this research contributes to the expanding corpus of knowledge on plant-based therapeutics, fostering the development of safer, more accessible, and efficacious treatment modalities. The findings of this study may pave the way for the integration of *M. pubescens* into modern medical practice, underscoring the significance of ethnobotanical research in drug discovery and development.

MATERIALS AND METHODS

Chemicals

Methanol and other reagents used for extraction and in vivo pharmacological evaluations were of laboratory grade, procured from Merck, Germany. Diazepam and loperamide were generously supplied by Incepta Pharmaceuticals Ltd., while Diclofenac Sodium was obtained from Beximco Pharmaceuticals Ltd., both based in Dhaka, Bangladesh. All other necessary chemicals were locally sourced through Taj Scientific Ltd., Chittagong, Bangladesh.

Material of the plant

The plant specimen of *M. pubescens* was obtained from the Hazarikhil Wildlife Sanctuary, Chattogram. It was identified by taxonomist Dr. Shaikh Bokhtiar Uddin, a professor in the University of Chittagong's Department of Botany. A specimen with the herbarium number SBU030524-30 was prepared and preserved.

Plant material preparation

After thorough cleaning, the harvested plant portion of *M. pubescens* leaves was air-dried for 15 days. After a complete air-drying procedure, the leaves were processed into a coarse powder using a grinding machine in the Department of Pharmacy, BGC Trust University, Bangladesh. After that, the powder was stored in an airtight container.

Plant material extraction

3.8 litres of pure methanol (Merck, Germany) were used to soak 700 grams of the powdered substance in a sterile, round-bottomed flask (5 litres). With periodic shaking and stirring, the reservoir and its contents were wrapped with foil and stored for 15 to 20 days. A new cotton plug and Whitman No. 1 filter paper were used to filter the whole mixture. A Buchii Rota (Cole-Parmer, UK) evaporator was used to lower the filtrate's volume under reduced pressure at a temperature below 53 ˚C. The extracted material weighed 10.04 grams [15].

Qualitative phytochemical screening

Preliminary phytochemical screening involves standard qualitative tests to detect various bioactive compounds in plant extracts. These tests produce visible reactions such as color changes or precipitation. Specific reagents are used to identify classes like alkaloids, flavonoids, carbohydrates, glycosides, phenolics, and tannins, providing a quick and effective assessment of the extract's phytochemical profile [16].

Experimental animals

Swiss male albino mice of five to six weeks of age, weighing about 20 - 30 g, were obtained from the animal facility of the Department of Pharmacy at the International Islamic University, Chittagong. They were housed properly and acclimatized under standard conditions for 1 week at the animal facility of BGC Trust University, Bangladesh. Environmental parameters included an ambient temperature of 25±2°C with 12 hours of light and dark cycles, having proper ventilation [17]. Mice were supplied

with a conventional laboratory diet and water. Before experimentation, animals were permitted to acclimate to the laboratory setting for 48 hours. The experimental protocol was authorized by the animal ethics committee of the institution (Reg. no. BGCTUB/AEAC/24/027).

Acute toxicity study

Using the previously mentioned methodology [18], an acute toxicity investigation was carried out. The effects of *M. pubescens* extracts were assessed in this study using groups of five Swiss albino mice each. The mice were fasted overnight before receiving oral doses of 1000, 2000, 3000, and 4000 mg/kg of the extracts. Post-administration, they were kept without diet for 3–4 hours. The mice were monitored for thirty minutes immediately after dosing, then for the first 24 hours, and once daily for the next 3 days. Key parameters, including physical appearance (skin, fur, eyes), vital signs (respiration, circulation), and neurological behaviour, were monitored for signs of toxicity. The lethal dose (LD_{50}) was estimated, and the effective dose (ED_{50}) was considered as one-tenth of the LD_{50}.

Investigation of analgesic activity

Abdominal constriction test

The analgesic effect was assessed using the acetic acid-induced writhing response method [19, 20]. Mice were injected with 0.7% acetic acid intraperitoneally to induce abdominal contractions (writhing), and writhing responses were monitored as an indicator of pain. A reduction in writhing compared to the control group indicates analgesic activity. Diclofenac served as a positive control. After oral administration of the test substances, control, and diclofenac, acetic acid was injected, and the number of writhing episodes was calculated over fifteen minutes, starting five minutes after injection.

Tail flick test

The central analgesic property of the extract was evaluated using the thermal tail immersion method [21]. Mice were placed in hot water (50 ± 10°C), and the time taken for them to withdraw their tails was recorded. Baseline readings were taken before administering the test, control, and reference samples. Mice were then observed at 30, 60, 90, and 120 minutes post-administration. The percentage increase in tail withdrawal time, relative to the control, was calculated, with a higher percentage indicating greater central analgesic activity. The effects of the test samples were compared to the control and standard, and the Maximal Possible Effect (MPE) was calculated using the following equation:

$$\%\ of\ MPE\ = \frac{Post\ drug\ latency\ -\ Pre\ drug\ latency}{Cut\ off\ time\ -\ Pre\ drug\ latency} \times 100$$

The percentage of time elongation was determined using the following equation:

$$\%\ of\ Elongation = \frac{Latency\ of\ Test\ Sample\ -\ Latency\ of\ Control}{Latency\ of\ Test} \times 100$$

Anxiolytic profiling

Anxiety in rodents is frequently evaluated using the Elevated Plus Maze (EPM) test, relying on their natural fear of heights and open spaces [22]. The maze consists of both open and enclosed arms, with animals allowed five minutes to explore. Rodents typically avoid the open arms, exhibiting fear responses such as freezing or defecating. Anxiolytic medications lengthen the time spent and the frequency of admissions into the open arms, whereas anxiogenic drugs shorten these behaviors. Anxiety levels are assessed based on the number of entries and duration spent in the open arms, with more exploration suggesting lower anxiety.

The apparatus is used to evaluate neophilia (curiosity) in rodents by observing head-dipping behavior in a confined area with floor holes [23]. Frequent head-dipping indicates high curiosity, while fewer dips suggest anxiety or low curiosity [21]. The number of head dips is inversely related to anxiety levels, with anxiolytic effects likely increasing head-dipping behavior [24]. Mice were placed on the board for a five-minute trial, and head-dips were counted for 30 minutes after dosing.

Sedative profiling

The apparatus used for the test has a floor area of around 0.5 square meters. The device rose to a height of 50 centimeters. After oral administration of the control (saline), standard (Diazepam), and test samples (200 and 400 mg/kg), the number of squares visited by the mice at 0, 30, 60, 90, and 120 minutes was noted [25]. The visits were tallied every 3 minutes.

A fixed wooden divider with a round hole (3.5 cm diameter, 7.5 cm height) was used to separate two chambers [26]. Mice were transferred between the chambers through the hole, and the number of crossings was recorded at 0, 30, 90, and 120 minutes in 3-minute intervals using a tally counter.

The anti-diarrheal effect of *M. pubescens* crude extract was tested using the castor oil-induced diarrhea model in mice [27]. Mice were first given test samples, standard (loperamide), or control (normal saline). After 30 minutes, 1 ml of castor oil was given to each mouse to cause diarrhea. The mice were observed for 4 consecutive hours, and the number of defecations was recorded for each hour. The results from the experimental groups were compared to those of the control group to assess the anti-diarrheal activity.

Statistical analysis

The results are presented as mean ± standard error of the mean (SEM). Statistical significance was determined using one-way ANOVA with Dunnett's test, where ***P < 0.001, **P < 0.01, and *P < 0.05 were considered significant. The analysis was performed using SPSS software (version 25).

RESULTS

Phytochemicals

The initial phytochemical investigations conducted on *M. pubescens* leaf extracts revealed the presence of a variety of Phytoconstituents, as detailed in the following Table 1 [22].

Table 1. Phytochemical analysis of the methanolic leaf extract of *M. pubescens*.

Phytochemicals	Result
Alkaloids	+++
Flavonoids	++
Reducing Sugar	+++
Carbohydrate	+
Glycosides	++
Cardiac Glycosides	++
Phenolic Compound	+++
Tannins	+++

"+" =Present, "++" =More presence, "+++" =Very much presence

Effect of methanolic extract of *M. pubescens* on acute toxicity in mice

Doses of the experimental extracts given to mice in the acute toxicity research did not cause any observable side effects (Table 2). The mice did not exhibit symptoms such as decreased motor activity, restlessness, seizures, loss of consciousness, diarrhoea, or excessive tear production. Moreover, none of the mice died during the experiment, irrespective of the dosage administered. As a result, it was determined that the LD_{50} of the extracts is higher than 4000 mg/kg, indicating a relatively low level of acute toxicity at the tested concentrations. This suggests that the extracts may have a favourable safety profile for further investigation (Table 2).

Table 2. Acute toxicity for the methanolic extract of *M. pubescens* in Swiss Albino mice.

Parameter	Observation
Extract Doses Administered	Up to 4000 mg/kg
Observed Side Effects	None
Motor Activity	Normal (no decrease observed)
Restlessness	Absent
Seizures	Absent
Loss of Consciousness	Absent
Diarrhea	Absent
Excessive Tear Production	Absent
Mortality	0% (no deaths at any dose)
Estimated LD_{50}	> 4000 mg/kg

Effect of methanolic extract of *M. pubescens* on analgesic activity in mice

The methanolic extract of *M. pubescens* leaves demonstrated significant analgesic activity in both peripheral (abdominal constriction test) and central (tail flick method) pain models. In the abdominal constriction test, the extract at 200 mg/kg showed 63.80% inhibition of writhing (38.66 ± 4.09 writhes; $P < 0.001$), while the 400 mg/kg dose exhibited 19.50% inhibition (53.00 ± 4.04 writhes). The standard Diclofenac (50 mg/kg) demonstrated the highest inhibition of 94.84% (5.33 ± 0.88 writhes), confirming the peripheral analgesic effect of the extract. In the tail immersion method, which evaluates the central analgesic effect, the 200 mg/kg extract significantly increased the reaction time to 4.58 ± 0.47 seconds at 120 minutes, showing 25.07% elongation of latency time, while the 400 mg/kg dose showed 4.01 ± 0.49 seconds (14.44% elongation). The standard Diclofenac (50 mg/kg) exhibited the highest response of 8.50 ± 0.20 seconds (65.0%

elongation; P < 0.001). Tables 3, 4, and 5 present an overview of the experimental results, illustrating the effects observed in the treated groups compared to the controls.

Table 3. Analgesic effect of *M. pubescens* leaves using the writhing test.

Animal Group	Number of Writhing (Mean ± SEM)	% Inhibition of Writhing
Control	63.33 ± 6.00	0
Standard	5.33 ± 0.88***	94.84
ME 200	38.66 ± 4.09***	63.80
ME 400	53 ± 4.04	19.50

Analgesic effect of the extracts of *M. pubescens* leaves on acetic acid-induced writhing test. Each value from the above table represents Mean±SEM (standard mean error). The statistical analysis was followed by using One-Way Analysis of Variance (Dunnett's test) where n=5, *p < 0.05, **P< 0.01, and ***p < 0.001 when compared with Control. Control= Normal Saline (10 ml/kg), Standard= Diclofenac (50 mg/kg), ME 200= Crude methanol extract of *M. pubescens* leaf extract (200 mg/kg), and ME 400= Crude methanol extract of *M. pubescens* leaf extract (400 mg/kg).

Table 4. Analgesic effect of *M. pubescens* leaves on the tail immersion test.

Test samples	Reaction times in seconds (Mean ± SEM)				
	Pre-treatment	30 minutes	60 minutes	90 minutes	120 minutes
Control	4.55 ±0.78	2.67 ± 0.19	3.09 ± 0.45	3.60 ± 0.24	3.43 ± 0.13
Standard	2.56±0.08	5.27 ± 0.23**	7.10±0.12*	7.76±0.18***	8.5±0.20***
ME-200	3.79 ± 0.51	3.33 ± 0.98	3.87 ± 0.96	4.42 ± 0.41	4.58 ± 0.47
ME-400	4.10 ± 1.30	2.91 ± 0.39	3.35 ± 0.21	3.97 ± 0.40	4.01 ± 0.49

Each value from the above table represents Mean±SEM (standard mean error). The statistical analysis was followed by using One-Way Analysis of Variance (Dunnett's test) where n=5, *p < 0.05, **P< 0.01, and ***p < 0.001 when compared with Control. Control= Normal Saline (10 ml/kg), Standard= Diclofenac (50 mg/kg), ME 200= Crude methanol extract of *M. pubescens* leaf extract (200 mg/kg), and ME 400= Crude methanol extract of *M. pubescens* leaf extract (400 mg/kg).

Table 5. Analgesic activity of *M. pubescens* leaves on the tail immersion test.

Test samples	% Elongation of latency time			
	30 minutes	60 minutes	90 minutes	120 minutes
Standard	50%	68.59%	63.9%	65.0%
ME200	19.92%	20.22%	18.60%	25.07%
ME400	8.46%	7.94%	9.38%	14.44%

Each value from the above table represents Mean±SEM (standard mean error). The statistical analysis was followed by using One-Way Analysis of Variance (Dunnett's test) where n=5, *p < 0.05, **P< 0.01, and ***p < 0.001 when compared with Control. Control= Normal Saline (10 ml/kg), Standard= Diclofenac (50 mg/kg), ME 200= Crude methanol extract of *M. pubescens* leaf extract (200 mg/kg), and ME 400= Crude methanol extract of *M. pubescens* leaf extract (400 mg/kg).

Effect of the methanol extract of *M. pubescens* on anxiolytic activity

The methanolic leaf extract of *M. pubescens* exhibited notable anxiolytic effects in both the EPM and Hole Board Test, demonstrating a dose-dependent reduction in anxiety-like behavior. In the EPM method, the 200 mg/kg dose significantly prolonged the time spent in the open arm (16.6 ± 4.40 seconds) while decreasing the number of entries into the closed arm (8 ± 1.52; P < 0.001). Similarly, the 400 mg/kg dose showed 15.33 ± 0.67 seconds in the open arm and 8.67 ± 0.67 entries in the closed arm. The standard Diazepam (1 mg/kg) exhibited the highest effect with 67.24 ± 3.39 seconds in the open arm and 7.33 ± 0.33 closed arm entries (P < 0.001). In the Hole Board Test, the 200 mg/kg dose increased the number of head dips to 26 ± 1.86, while the 400 mg/kg dose showed 27 ± 3.51 head dips, compared to the control group (18 ± 2 head dips). The standard Diazepam (1 mg/kg) showed the highest effect with 69 ± 1.15 head dips (P < 0.001). These were shown in Tables 6 and 7.

Table 6. Anxiolytic activity of *M. pubescens* in the open and closed arms of the EPM

Group	Open Arm (Mean ± SEM)		Closed Arm (Mean ± SEM)	
	Time Spent (sec.)	Number of Entries	Time Spent (sec.)	Number of Entries
Control	6.33 ± 1.76	3 ± 0.57	115.33 ± 3.38	15.33 ± 3.38
Standard	67.24 ± 3.39***	15.33 ± 0.88***	117.15 ± 1.33***	7.33 ± 0.33***
ME-200	16.6 ± 4.40	1.67 ± 0.66	115.67 ± 8.21***	8 ± 1.52***
ME-400	15.33 ± 0.67	1.33 ± 0.33	117 ± 13.11***	8.67 ± 0.67***

Anxiolytic activity of methanolic extract of *Mussaenda pubescens* by counting the mean time spent and number of entries of mice in the open and closed arms on the Elevated Plus Maze (EPM). Each value from the above table represents Mean±SEM (standard mean error). The statistical analysis was followed by using One-Way Analysis of Variance (Dunnett's test) where n=5, *$p < 0.05$, **$P < 0.01$, and ***$p < 0.001$ when compared with Control. Control= Normal Saline (10 ml/kg), Standard= Diazepam (1 mg/kg), ME 200= Crude methanol extract of *M. pubescens* leaf extract (200 mg/kg), and ME 400= Crude methanol extract of *M. pubescens* leaf extract (400 mg/kg).

Table 7. Anxiolytic activity of *M. pubescens* in the Hole-board Test.

Group	Number of Head Dip (Mean ± SEM)
Control	18 ± 2
Standard	69 ± 1.15***
ME-200	26 ± 1.86
ME-400	27 ± 3.51

Anxiolytic activity of *Mussaenda pubescens* by counting the mean number of head dips in the Hole-board Test. Each value from the above table represents Mean±SEM (standard mean error). The statistical analysis was followed by using One-Way Analysis of Variance (Dunnett's test) where n=5, *$p < 0.05$, **$P < 0.01$, and ***$p < 0.001$ when compared with Control. Control= Normal Saline (10 ml/kg), Standard= Diazepam (1 mg/kg), ME 200= Crude methanol extract of *M. pubescens* leaf extract (200 mg/kg), and ME 400= Crude methanol extract of *M. pubescens* leaf extract (400 mg/kg).

Effect of the methanol extract of *M. pubescens* on CNS depressant activity

The methanolic extract of *M. pubescens* leaves exhibited significant CNS depressant activity in a dose-dependent manner, as assessed by the Open Field Test (OFT) and the Hole Cross Test (HCT). In the Open Field Test, the 200 mg/kg dose reduced the number of movements from 12 ± 2.96 at 0 minutes to 3 ± 1.52 at 120 minutes ($P < 0.001$), while the 400 mg/kg dose showed a reduction from 16 ± 1 to 8.67 ± 0.88 ($P < 0.001$). The standard Diazepam (1 mg/kg) exhibited the highest reduction, from 58 ± 10.44 to 15.67 ± 1.67 ($P < 0.001$). In the Hole Cross Test, the 200 mg/kg dose significantly decreased movements from 6.33 ± 2.02 to 1.33 ± 1.33 ($P < 0.001$), while the 400 mg/kg dose reduced movements from 5.33 ± 2.90 to 3 ± 0.58 ($P < 0.05$). The standard Diazepam showed the highest reduction from 10.33 ± 0.89 to 1.66 ± 0.33 ($P < 0.001$). These were shown in Tables 8 and 9.

Table 8. Open Field Test for CNS depressant activity of *M. pubescens*

Animal Group	Number of Movements (Mean ± SEM)				
	0 Minute	30 Minutes	60 Minutes	90 Minutes	120 Minutes
Control	61.67 ± 5.48	59.33 ± 4.91	59 ± 4.93	50 ± 5.36	46.33 ± 3.38
Standard	58 ± 10.44	39 ± 5.19***	25 ± 2.89***	20 ± 0.58***	15.67 ±1.67***
ME-200	12 ±2.96***	10.67 ± 2.84***	7.67 ± 1.85***	5 ± 1.73***	3 ± 1.52***
ME-400	16 ± 1***	13.67 ± 2.02***	11 ± 1.52***	9.67 ± 1.45***	8.67 ± 0.88***

Each value from the above table represents Mean±SEM (standard mean error). The statistical analysis was followed by using One-Way Analysis of Variance (Dunnett's test) where n=5, *$p < 0.05$, **$P < 0.01$, and ***$p < 0.001$ when compared with Control. Control= Normal Saline (10 ml/kg), Standard= Diazepam (1 mg/kg), ME 200= Crude methanol extract of *M. pubescens* leaf extract (200 mg/kg), and ME 400= Crude methanol extract of *M. pubescens* leaf extract (400 mg/kg).

Table 9. CNS depressant activity of *M. pubescens* in the hole cross test.

Animal Group	Number of Movements (Mean ± SEM)				
	0 Minute	30 Minutes	60 Minutes	90 Minutes	120 Minutes
Control	12 ± 0.58	10.67± 0.33	9.33 ± 0.33	7.33 ± 1.20	5 ± 0.58
Standard	10.33 ± 0.89	7.33 ± 0.33	5 ± 0.58*	3.33 ± 0.89*	1.66 ± 0.33
ME-200	6.33 ± 2.02	4.33 ± 2.02*	2.67±1.20***	1.33 ± 0.33**	1.33 ± 1.33*
ME-400	5.33 ± 2.90	6.67 ± 2.02*	3.67± 0.88***	3.33 ± 1.20*	3 ± 0.58

Screening of CNS depressant activity of *Mussaenda pubescens* by calculating the mean number of movements of mice in the hole cross test. Each value from the above table represents Mean±SEM (standard mean error). The statistical analysis was followed by using One-Way Analysis of Variance (Dunnett's test) where n=5, *$p < 0.05$, **$P < 0.01$, and ***$p < 0.001$ when compared with Control. Control= Normal Saline (10 ml/kg), Standard= Diazepam (1 mg/kg), ME 200= Crude methanol extract of *M. pubescens* leaf extract (200 mg/kg), and ME 400= Crude methanol extract of *M. pubescens* leaf extract (400 mg/kg).

Effect of the methanol extract of *M. pubescens* on antidiarrheal activity

The methanolic leaf extract of *M. pubescens* demonstrated significant antidiarrheal activity in a dose-dependent manner, evaluated using the castor oil-induced diarrhea model. The 200 mg/kg dose reduced the total number of defecations to 4.67 ± 0.67, resulting in a 43.94% inhibition, with complete (100%) inhibition observed during the second hour. On the other hand, the 400 mg/kg dose demonstrated a greater reduction (3.33 ± 1.67 defecations) with 60.02% inhibition and 100% inhibition in the fourth hour. The standard loperamide (50 mg/kg) exhibited the highest inhibition of 60.02%, completely halting defecation in the fourth hour (100%). These were shown in Table 10.

Table 10. Antidiarrheal activity of crude methanol extract of *M. pubescens* by using the castor oil-induced diarrhea test.

Groups	Mean ± SEM (% of Inhibition)				
	1st h	2nd h	3rd hr.	4th h	Total
Control	3.67 ±1.76	2.33 ±0.33	2.00 ±1.00	2.00 ±1.00	8.33 ±0.88
Standard	1.00 ±0.58 (72.75%)	1.67 ±0.88 (28.32%)	1.00 ±0.00 (50%)	0.00 ±0.00 (100%*)	3.33 ±0.67 (60.02%)
ME-200	2.67 ±1.20 (27.25%)	0.00 ±0.00 (100%*)	1.33 ±1.33 (33.5%)	0.67 ±0.33 (66.5%)	4.67 ±0.67 (43.94%)
ME-400	0.67 ±0.67 (81.74%)	1.00 ±0.58 (57.08%)	1.67 ±0.67 (16.55%)	0.00 ±0.00 (100%*)	3.33 ±1.67 (60.02%)

Screening of antidiarrheal activity of crude methanol extract of *Mussaenda pubescens* by using castor oil-induced diarrhea test. Each value from the above table represents Mean±SEM (standard mean error). The statistical analysis was followed by using One-Way Analysis of Variance (Dunnett's test) where n=5, *$p < 0.05$, **$P < 0.01$, and ***$p < 0.001$ when compared with Control. Control= Normal Saline (10 ml/kg), Standard= Loperamide (50 mg/kg) , ME 200= Crude methanol extract of *M. pubescens* leaf extract (200 mg/kg, and ME 400= Crude methanol extract of *M. pubescens* leaf extract (400 mg/kg).

DISCUSSION

The present analysis aimed to investigate the pharmacological properties of the methanolic extract of *M. pubescens* leaves, a plant traditionally used for various medicinal purposes. The extract was evaluated for its anxiolytic, analgesic, CNS depressant, and anti-diarrheal activity, with promising results observed across these areas.

Regarding anxiolytic effects, in both cases, the methanolic extract of *M. pubescens* considerably enhanced the duration of time spent in the open arms of the EPM and the frequency of head dipping in the HBT. These behaviors are well-established markers of reduced anxiety, as increased exploration in unprotected spaces typically indicates lower levels of anxiety [28]. The dose-dependent nature of this response, particularly at 400 mg/kg, suggests a gradual increase in efficacy. These findings are consistent with the

presence of phytochemicals like flavonoids and alkaloids in the plant extract [29], which have been previously reported to modulate GABAergic pathways, known for their role in anxiety regulation [30]. In the abdominal constriction test, the methanolic extract significantly decreased the number of writhing movements (63.8% inhibition), a result comparable to diclofenac sodium, a standard anti-inflammatory drug. This suggests that the extract may exert peripheral analgesic effects, possibly by inhibiting the synthesis of pro-inflammatory mediators like PGE2 and PGF2α [31]. Furthermore, the tail immersion test confirmed the central analgesic properties of the extract, as evidenced by the increased reaction time to thermal stimuli, indicative of central pain modulation. This dual mode of action, both peripheral and central, aligns with the analgesic mechanisms of many non-opioid pain relievers [32].

CNS depressant activity was also observed in this study, with the methanolic extract significantly reducing locomotor activity in the hole-cross and open field tests. This reduction in exploratory behavior is a typical indicator of sedative or hypnotic effects, suggesting that the extract may possess CNS depressant properties [33]. The involvement of GABAergic systems in this effect is plausible, as GABA is the principal inhibitory neurotransmitter in the central nervous system [34]. Many sedative compounds, especially those derived from plants, exert their effects by enhancing GABAergic inhibition [35]. The flavonoids and tannins present in *M. pubescens* are likely contributing to these sedative effects, similar to other plant-derived compounds known to modulate GABA receptors.

The anti-diarrheal potential of the methanolic extract was evaluated using the castor oil-induced diarrhea model, a well-established assay for screening anti-diarrheal agents. The extract significantly reduced both diarrhea incidence and intestinal fluid accumulation in a dose-dependent manner [2]. The tannins and flavonoids in the extract are likely responsible for these effects, as these compounds are known for their anti-inflammatory and anti-secretory properties. The mechanism may involve inhibition of ricinoleic acid-induced prostaglandin release or modulation of intestinal motility, which can reduce fluid secretion and enhance water absorption in the colon [36]. Although the anti-diarrheal effects were less potent than the standard drug loperamide, the observed activity supports the potential of *M. pubescens* as a treatment for diarrhea, particularly in resource-limited settings where traditional remedies are commonly used [37].

In conclusion, the methanolic extract of *M. pubescens* leaves exhibits a wide range of pharmacological activities, including anxiolytic, analgesic, CNS depressant, and anti-diarrheal effects. These results validate its traditional use in folk medicine and suggest its potential as a source of novel therapeutic agents. However, as these findings are preliminary, further studies are required to isolate the specific bioactive compounds responsible for these effects and to conduct more detailed in vivo studies, including clinical trials, to assess the safety, efficacy, and potential side effects of the extract. Bioactivity-guided fractionation and molecular mechanistic studies are essential for understanding the exact pharmacological pathways involved and for developing safe and effective plant-based therapies.

CONCLUSIONS

The methanolic extract of *M. pubescens* leaves exhibits a promising spectrum of pharmacological activities, including anxiolytic, analgesic, CNS depressant, and anti-diarrheal effects, reinforcing its traditional therapeutic use. These findings highlight its potential as a valuable source for the development of novel plant-based remedies. However, further research is essential to isolate the active compounds, understand their

underlying mechanisms, and assess the safety and efficacy of the extract in clinical settings, paving the way for its future medicinal applications.

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AUTHOR CONTRIBUTIONS

THF, UTN, and MZU jointly planned and designed the research. UTN and MMR arranged the facilities and supervised the study. THF, TN, and MMM conducted all laboratory work. THF, UTN, TN, MMM, and MMR contributed to the study design and data interpretation, with efforts focused on statistical analysis. THF, UTN, MZU, AKN, NNM, RB, and SR participated in drafting the manuscript and thoroughly checked and revised it for formatting, grammar, and language standards. All authors reviewed and approved the final version of the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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