JoP Journal of Pharmacopuncture

Journal of Pharmacopuncture 2024;27(3):234-244
pISSN: 2093-6966 • eISSN: 2234-6856
https://doi.org/10.3831/KPI.2024.27.3.234

Original Article

Anxiolytic and Antianhedonic-like Effects of *Psidium guajava* Leaf in Alcohol-Withdrawn Mice

Vanjarapu Harithadevi[1], Vijayapandi Pandy[1,2]*

[1]Department of Pharmacology, Chalapathi Institute of Pharmaceutical Sciences, Chalapathi Nagar, Lam, Guntur, Andhra Pradesh, India
[2]Department of Pharmaceutical Sciences, School of Health Sciences and Technology, Dr. Vishwanath Karad MIT World Peace University (MIT-WPU), Pune, Maharashtra, India

Received April 6, 2024
Reviewed April 15, 2024
Accepted July 8, 2024

***Corresponding Author**
Vijayapandi Pandy
Department of Pharmaceutical Sciences, School of Health Sciences and Technology, Dr. Vishwanath Karad MIT World Peace University (MIT-WPU), 124 Paud Road, Kothrud, Pune 411038, India
Tel: +91-20-2570-3400
E-mail: pandiphd@gmail.com; vijayapandi.pandy@mitwpu.edu.in

Objectives: Alcohol withdrawal syndrome manifests through a range of symptoms, including anxiety and anhedonia, significantly affecting the quality of life of those affected. This study investigates the potential therapeutic effects of the methanolic extract of *Psidium guajava* leaves (MPG) on anxiety and anhedonia in Swiss albino female mice undergoing alcohol withdrawal.
Methods: Four groups of mice underwent alcohol withdrawal, with one group undergoing saline withdrawal as a control. On the test day, behavioral assessments were conducted to evaluate anxiety and anhedonia. Groups I and II received sodium carboxymethylcellulose, Group III received diazepam, and Groups IV and V received varying oral doses of MPG.
Results: The results indicate significant anti-anhedonic and anxiolytic effects of MPG. These effects were observed through changes in parameters measured in the Open Field test, Elevated Plus Maze test, Marble Burying test, and Sucrose Preference test. Mice treated with MPG displayed reduced anxiety-like behaviors and increased sucrose preference compared to untreated mice undergoing alcohol withdrawal.
Conclusion: These findings suggest that *Psidium guajava* leaf extract may have therapeutic potential in alleviating anxiety and anhedonia associated with alcohol withdrawal. The observed effects indicate that MPG could serve as a promising adjunct therapy for managing alcohol withdrawal symptoms, thereby enhancing the overall well-being of individuals undergoing alcohol cessation.

Keywords: elevated plus maze, marble burying test, open field test, *Psidium guajava*, sucrose preference test

INTRODUCTION

The most commonly used and abused substance worldwide is alcohol. According to the World Health Organization, alcohol use contributes to the deaths of 3 million people annually, along with numerous disabilities and illnesses. Globally, hazardous alcohol consumption accounts for 5.1% of the disease burden, with men and women experiencing burdens of 7.1% and 2.2%, respectively [1]. Alcohol use is the leading risk factor for premature disability and mortality and is responsible for 10% of deaths among individuals, aged 15 to 49 years.

Alcohol withdrawal syndrome (AWS) is a well-recognized condition that arises when heavy or frequent drinking is abruptly stopped, whether intentionally or inadvertently [2]. Approximately 18% of the general population is estimated to experience alcohol use disorder (AUD) during their lifetime, with an annual prevalence of 5%. AWS, associated with infectious complications and increased mortality, affects 8% to 40% of patients in surgical intensive care units (ICUs) and over 20% of adults in emergency departments. Up to half of the individuals with AUD experience withdrawal symptoms, which result from various neurochemical mechanisms. Notably, the increased inhibitory effect of gamma-aminobutyric acid (GABA) and reduced excitatory effect of N-methyl-D-aspartate (NMDA)

receptors in the presence of alcohol are reversed upon its cessation. AWS is mediated by decreased GABAergic system responsiveness and increased glutamatergic system responsiveness in the brain [3]. Consequently, most individuals with AWS exhibit typical symptoms including anxiety, irritability, agitation, tremors, and signs of increased adrenaline release, as well as severe conditions, such as delirium tremens (DT) and withdrawal seizures [3].

Currently, benzodiazepines (BZDPs) are the only effective treatment and preventive measure for all severities of AWS [4]. However, BZDPs have significant neurological, physiological, and cognitive side effects. Consequently, there is a pressing need for new, effective medications with minimal adverse effects to manage AWS. Natural products are a primary source for discovering novel agents for treating various conditions, including neurological disorders, such as anxiety, depression, psychosis, and substance abuse.

Psidium guajava Linn, commonly known as guava, is an ancient medicinal plant widely used in Asian countries. Renowned for its therapeutic properties in treating both infectious and non-infectious diseases, *Psidium guajava* has been historically used to treat jaundice, dyspepsia, gastrointestinal discomfort, stomach pain, diarrhea, and edema [5]. Additionally, it exhibits diverse pharmacological effects on the neurological, digestive, and cardiovascular systems [6]. The present study employed mouse behavioral tests, such as elevated plus maze (EPM), sucrose preference test (SPT), open field test (OFT), and marble burying test (MBT) to investigate the potential therapeutic effects of *Psidium guajava* Linn leaf methanolic extract in alleviating AW-induced anhedonia and anxiety in mice.

MATERIALS AND METHODS

1. Plant collection

Psidium guajava leaves were gathered in Rajahmundry, Andhra Pradesh, India's East Godavari district. The leaves were verified by a botanist from Guntur's Department of Botany and Microbiology at the Acharya Nagarjuna University, and a voucher specimen was deposited.

2. Extraction preparation

The method used for extracting the *Psidium guajava* leaf powder involved maceration. Initially, 40 grams of the powder was soaked in 200 mL of 75% v/v methanol and placed on a magnetic stirrer for 3 days. On the fourth day, the methanolic extract of *Psidium guajava* leaves (MPG) was obtained by filtering the mixture through Whatman filter paper (No. 1). The filtered extract was then evaporated to dryness at a temperature of 37℃. Finally, the percentage yield of the extract was calculated based on the amount of dry extract obtained relative to the initial weight of the leaf powder-methanol mixture.

3. Making 20% ethanol (v/v) with fomepizole

Ethanol 20 mL (99.9% (v/v)) was combined with 0.1 mL of fomepizole (4-methyl pyrazole), and the mixture was diluted in saline (79.9 mL).

4. Animals

We utilized a total of sixty-one female albino mice (Swiss), aged 12-14 weeks, weighing between 25 grams and 30 grams. The animals were acclimatized for 7 days at 24℃ before testing, during which they were habituated to handling. Throughout this period and during the study, the mice had *ad libitum* access to fresh water and rodent pellet food. Shredded tissue paper was provided as environmental enrichment for the animals. The experimental procedure was evaluated and approved by the Institutional Animal Ethics Committee (IAEC) of the Chalapathi Institute of Pharmaceutical Science, Guntur, following the criteria established by the CCSEA located in India's New Delhi (Approval number: O6/IAEC/CLPT/2022-2023).

5. Chemicals and drugs

The substances used in the study included ethanol obtained from Changshu Hong Sheng Fine Chemical Co., Ltd., fomepizole (4-methyl pyrazole) sourced from Sigma-Aldrich, 0.9% w/v sodium chloride injection I.P. manufactured by Otsuka Pharmaceutical India Private Limited, and diazepam injection I.P. provided by Neon Laboratories Limited.

6. Phytochemical analysis

The methanolic extract of *Psidium guajava* leaves (MPG) test sample was prepared at a concentration of 10 mg/mL in methanol for phytochemical analysis. Various phytoconstituents present in MPG underwent standard identification tests as follows

[7]. Alkaloids were identified using the Hager and Wagner methods. Flavonoids were detected using the FeCl3 and lead acetate methods. Glycosides were tested using the Bontrager and Keller–Kiliani methods. Lipids and steroids were analyzed using the Liberman-Burchard and Salkowski methods. Tannins were identified using the FeCl3 and match stick methods. Volatile oils and fixed oils were assessed using the Spot and foam methods. Proteins were detected using the ninhydrin and biurets methods. These methods were employed to ascertain the presence of specific phytochemical groups within the MPG extract, providing a comprehensive profile of its chemical composition [7].

7. Experimental design

Initially, a dose-finding preliminary study was conducted where *Psidium guajava* leaves' methanolic extract (MPG) was administered at various oral doses (100, 200, and 500 mg/kg, 1 hour prior), alongside 1% w/v carboxymethyl cellulose (CMC, 1 mL/100 g body weight, orally, 1 hour prior), and diazepam (reference drug; 1 mg/kg intraperitoneally, 30 minutes prior). The anxiolytic activity was evaluated in groups of mice (n=5-6) using the OFT and EPM. Healthy animals were randomly assigned to each group. Based on this study, the minimum effective oral doses of MPG (100 and 200 mg/kg) were selected for subsequent alcohol withdrawal (AW) studies.

The primary objective of the current research was to investigate how MPG affects symptoms of AW, such as anxiety and anhedonia, in mice. To induce AW, all AW groups (Groups II to V) received intraperitoneal injections of 20% v/v ethanol solution twice daily for three days to prolong withdrawal effects, including anxiety and depression (first dose at 7 a.m. and second dose at 5 p.m.). Group I (control saline) underwent a similar treatment regimen but received saline injections, instead of ethanol. The animals were divided into five groups during the experimental alcohol/saline withdrawal phase, with each group consisting of 6-7 mice.

On the fourth day of withdrawal, Groups I (control saline) and II (ethanol-control) received 1% w/v carboxymethyl cellulose orally (CMC). Group III (reference control) received intraperitoneal diazepam at 1 mg/kg. *Psidium guajava* leaves' methanolic extract (MPG) was administered orally to Groups IV and V (test groups) at 100 mg/kg and 200 mg/kg, respectively. Behavioral assessments of AW symptoms in mice, including MBT, SPT, OFT, and EPM, were conducted one hour after oral dosing or 30 minutes after intraperitoneal injection, focusing on anxiety and depression. All behavioral parameters were evaluated by an observer blinded to the treatment protocol.

1) OFT

In the OFT, the test box is divided into two compartments: central and peripheral. Each square within the peripheral compartment measures 10 cm by 10 cm, similar to each square within the central compartment. The overall dimensions of the OFT box were 50 cm × 50 cm × 15 cm. During the test, the animal explored the OFT box for five minutes. The number of crossings (moving from one square to another) and the time spent in both the central and peripheral compartments were recorded. Additionally, the time spent specifically in the central compartment and the percentage of total square crossings that occurred in the central area, were calculated. The OFT trials were conducted in an environment illuminated by 150-lux white light and conducted outdoors, according to the referenced studies [1, 8].

2) EPM

The EPM consists of two open arms and two closed arms, each measuring 30 cm × 7.5 cm, positioned opposite each other. The center square where the arms intersect measured 7.5 cm. The maze was elevated 40 cm above the ground surface. During the EPM test, each mouse was placed in the center of the maze facing one of the open arms. The mice were allowed to freely explore the maze five minutes. Throughout this time, the number of entries and the duration spent in both the open and closed arms were recorded. A soft red light was used during the EPM study, providing illumination conditions as specified in the referenced studies [1, 9].

3) MBT

Each mouse was housed individually in a plastic cage filled with 5 cm deep husk bedding. The case measured 38 cm × 21 cm × 14 cm. On top of the bedding, there were 27 small glass marbles arranged in three rows and nine columns. The marbles had diameters of 10 mm and 12 mm. During the test, the animal was allowed to explore the cage for 5 minutes. After this period, any marbles that remained uncovered by the bedding were counted. A marble was considered "buried" if at least two-thirds (2/3) of its surface area was covered by husk bedding. Previous studies have found that anxiolytic medications typically reduce the number of marbles buried, as burying marbles

 https://doi.org/10.3831/KPI.2024.27.3.234

is indicative of anxiety-like behaviors in animals [1, 10].

4) SPT

During the SPT, each mouse were provided with two bottles (one containing regular drinking water and the other containing a 1% sucrose solution w/v) in its cage for 24 hours. No food was provided during the 24-hour study period. Just before the SPT commenced and again 12 hours into the study, the test animals were administered their respective medications.

After the initial 12-hour period, the positions of the bottles were switched to avoid any side preference effects. Following the full 24-hour period, the amounts of sucrose solution and

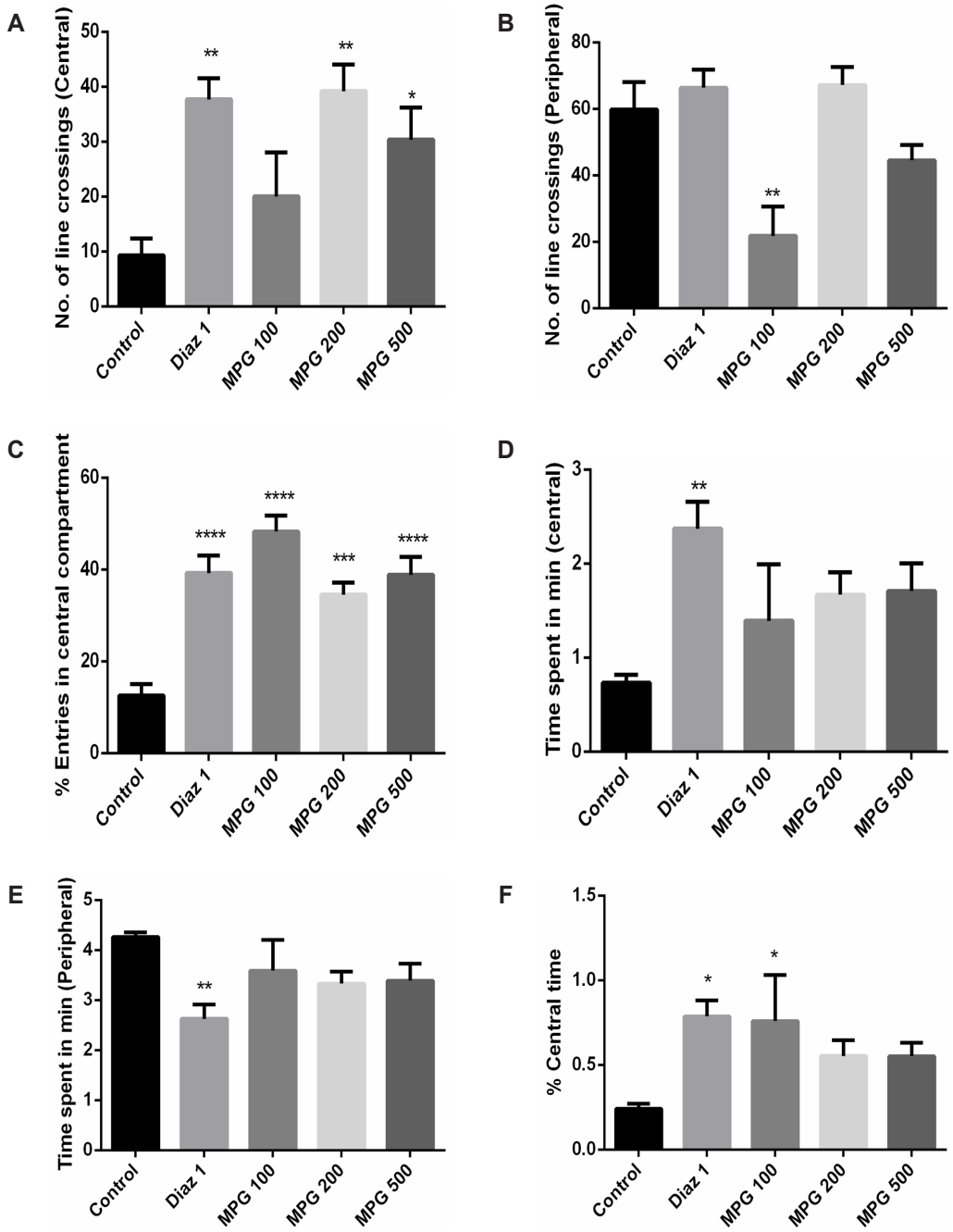

Figure 1. Effect of oral MPG (100, 200, and 500 mg/kg) on (A) Central line crossings, (B) Line crossings in the outskirts, (C) Crossings of the central line (%), (D) Total central time devoted, (E) Time spent in outskirts, (F) % of the time that mice (N = 5-6) spent in the center of an open field test. Statistical significance at *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 when compared with the control (vehicle) group.

water consumed by each mouse were measured. The sucrose preference ratio was then calculated using the formula:

$$\text{Sucrose consumption ratio} = \frac{\text{Sucrose intake/B.wt in kg}}{\text{Sucrose intake/B.wt in kg} + \text{Water intake/B.wt in kg}}$$

This ratio indicates the preference for the sucrose solution relative to water, which is used as a measure of anhedonia or reduced pleasure-seeking behaviors in mice [1].

8. Statistical analysis

Dunnett's multiple comparison tests were performed after a one-way analysis of variance (ANOVA) using GraphPad Prism 5. Significance was set at $p < 0.05$. All data were presented as mean ± SEM except for the SPT results, which were presented as a ratio.

RESULTS

1. MPG phytochemical analysis

Alkaloids, steroids, saponins, lipids, flavonoids, phenols, fixed oils, and volatile oils were all detected in the phytochemical studies. However, glycosides and cardiac glycosides were not detected.

2. Evaluation of the anxiolytic properties of *Psidium guajava* leaf methanolic extract in mice

1) OFT

The results of the one-way ANOVA with Dunnett's multiple comparison tests revealed significant differences among the treatment groups for several behavioral parameters in the OFT (Fig. 1A-F) including (A) the number of central line crossings ($F[4,24] = 5.939$, $p < 0.0018$), (B) the number of peripheral line crossings ($F[4,24] = 7.811$, $p < 0.0004$), (C) the percentage of central entries ($F[4,24] = 15.88$, $p < 0.0001$), (D) central time ($F[4,24] = 3.544$, $p < 0.0208$), (E) peripheral time $F[4,24] = 3.195$, $p < 0.0308$), and (F) % central time ($F[4,24] = 3.030$, $p < 0.0372$). MPG (at doses of 100 mg/kg, 200 mg/kg, and 500 mg/kg, orally) significantly increased the percentage of entries into the central compartment compared to the control group. These findings suggest that the anxiolytic effect of MPG was similar to the reference drug, diazepam (1 mg/kg, intraperitoneally). In the OFT, rodents typically exhibit less exploratory behaviors in the central compartment due to inherent anxiety, preferring instead to stay near the edges (thigmotaxis behavior). In this study, anxiolytic drugs, including MPG, promote increased exploration of the central compartments and reduce thigmotaxis behavior. These findings underscore the potential anxiolytic properties of MPG observed in normal animals, as evidenced by behavioral changes in the OFT parameters measured.

2) EPM

The results of the one-way ANOVA with Dunnett's multiple

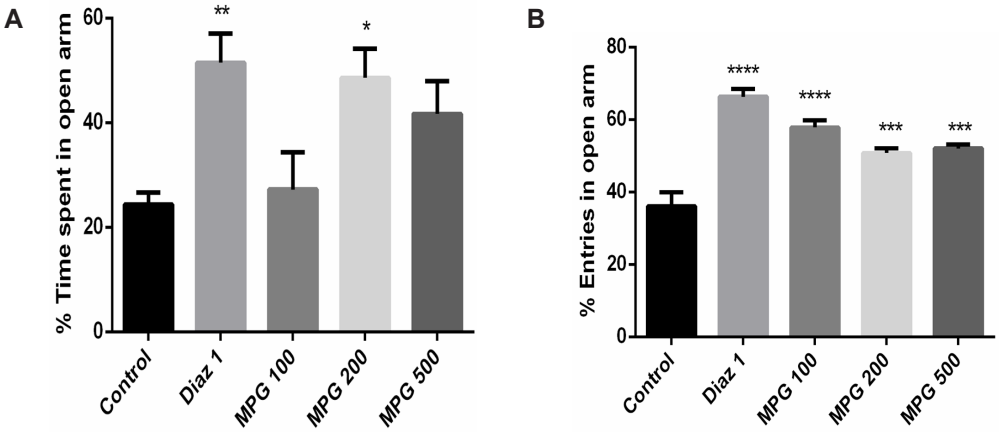

Figure 2. Effect of MPG oral doses (100, 200, and 500 mg/kg) on (A) % open arm time spent, (B) % open arm entries in the elevated plus maze test in mice (N = 5-6). Statistical significance at *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ when compared with the control (vehicle) group.

 https://doi.org/10.3831/KPI.2024.27.3.234

comparison tests revealed significant differences among the treatment groups for parameters in the EPM as follows (Fig. 2A, B): (A) percentage of open arm time spent ($F[4,24] = 5.021$, $p < 0.0044$) and (B) percentage of open arm entries ($F[4,24] = 22.86$, $p < 0.0001$). MPG (100 mg/kg, 200 mg/kg, and 500 mg/kg administered orally) significantly increased the percentage of entries into the open arms compared to the control group. These results suggest that the anxiolytic effect of MPG was similar to that observed with diazepam (1 mg/kg, intraperitoneally). In the EPM test, normal animals typically spend less time in the open arms due to fear of open spaces and the elevated height of the open arms above the floor. Anxiolytic drugs are known to increase exploratory behaviors in the open arms. In this study, MPG use increased the mice's exploratory behaviors in the open arms, reflecting reduced

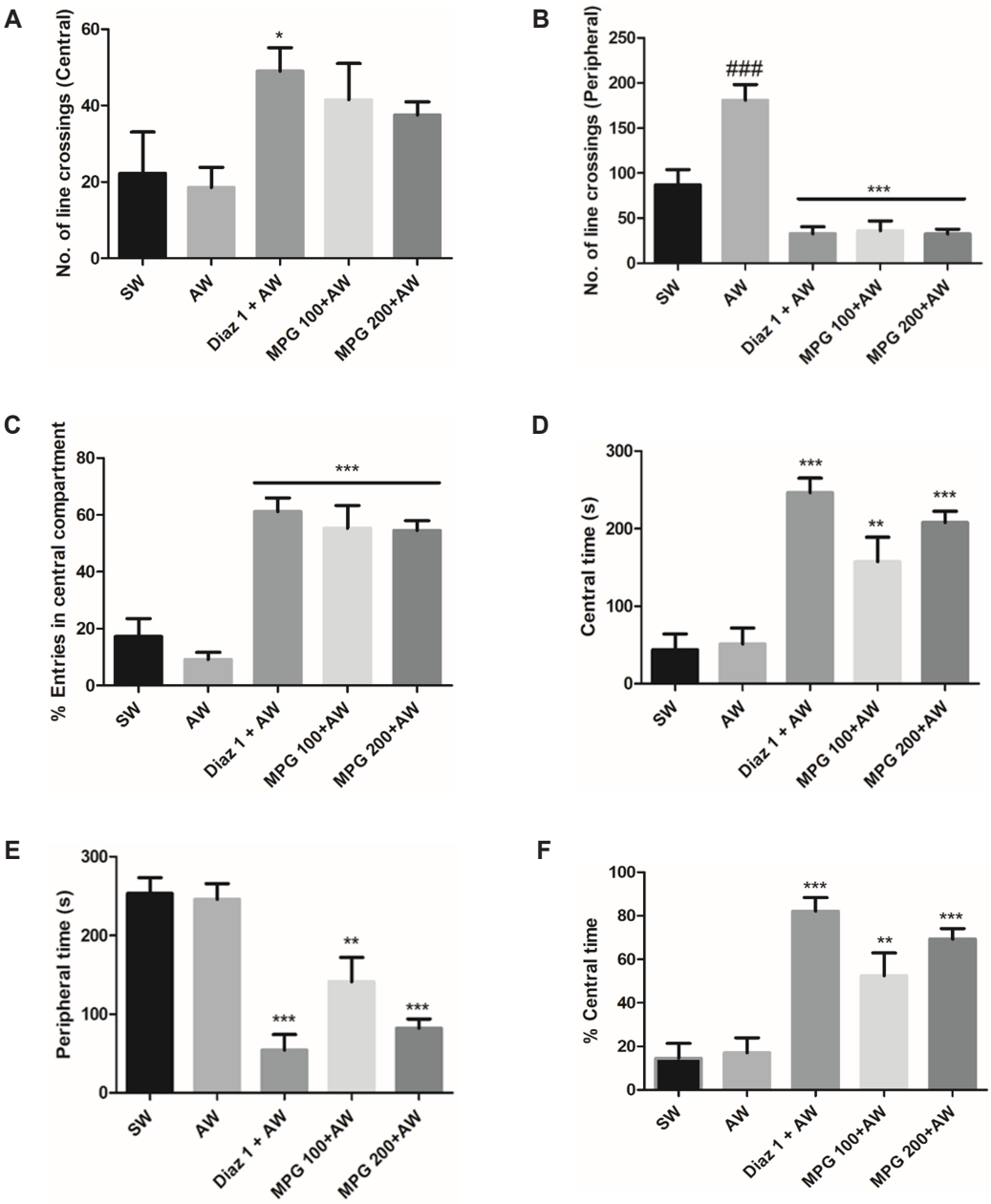

Figure 3. Effects of oral MPG (100 and 200 mg/kg) on (A) Central line crossings, (B) Line crossings along the outskirts, (C)% of line crossings (central), (D) Total amount of time spent (central), (E) Time spent in the outskirts, (F) % of the time spent (central) in the open field test in AW mice (N = 6-7). Statistical significance at *p < 0.05, **p < 0.01, and ***p < 0.001 when compared with the alcohol withdrawal control (AW) group; ###p<0.001 when compared with the saline withdrawal control (SW) group.

anxiety-like behavior in the animals. These findings highlight the potential anxiolytic properties of MPG in normal animals, as evidenced by changes in the EPM parameters measured.

3. Evaluation of the anxiolytic effect of *Psidium guajava* leaf methanolic extract in alcohol withdrawal mice

1) OFT

The results of the one-way ANOVA with Dunnett's multiple comparison tests revealed significant differences among the treatment groups for several behavioral parameters in the experimental setup (Fig. 3A-F): (A) the number of central line crossings ($F[4,27] = 2.837$, $p < 0.0438$); (B) the number of peripheral line crossings ($F[4,27] = 24.54$, $p < 0.0001$); (C) percentage of central entries ($F[4,27] = 19.93$, $p < 0.0001$), (D) time spent in central area ($F[4,27)] = 18.31$, $p < 0.0001$), (E) time spent in peripheral area ($F[4,27] = 18.32$, $p < 0.0001$), and (F) percentage of central time ($F[4,27] = 18.32$, $p < 0.0001$). AW mice exhibited a significant increase in the number of peripheral line crossings compared to the saline withdrawal mice, indicating heightened excitability and increased thigmotaxis behavior (Fig. 3B). Oral administration of MPG (at doses of 100 mg/kg and 200 mg/kg) markedly reduced the excitability behaviors observed in AW, similar to the effects seen when diazepam was administered intraperitoneally (1 mg/kg) (Fig. 3B). Furthermore, oral administration of MPG (100 mg/kg and 200 mg/kg) significantly increased the percentage of time spent in the central area and the number of entries into the central compartment, comparable to the effects observed with 1 mg/kg intraperitoneal diazepam (Fig. 3C, F). These findings demonstrated that MPG exhibited similar anxiolytic effects as diazepam in AW mice, as evidenced by improvements in central exploration and reduced thigmotaxis behavior.

These results demonstrate the potential therapeutic benefits of MPG in alleviating anxiety-related behaviors associated with AW. Its role as an anxiolytic agent in this experimental model was confirmed.

2) EPM

The results of the one-way ANOVA revealed significant effects among the treatment groups for the following parameters in the EPM (Fig. 4): (A) percentage of time spent in open arms ($F[4,27] = 83.86$, $p < 0.0001$) and (B) percentage of entries into open arms ($F[4,27] = 18.62$, $p < 0.0001$). Compared to the saline withdrawal mice, AW animals did not exhibit any anxiogenic-like activity during the EPM test. However, mice undergoing AW and treated orally with MPG at doses of 100 mg/kg and 200 mg/kg spent significantly more time in the open arms and made more entries into the open arms, compared to untreated mice. These findings indicated that MPG exerted an anxiolytic-like effect on AW mice, as evidenced by increased exploration of the open arms in the EPM. Taken together, these findings showed that MPG mitigated anxiety-related behaviors typically associated with AW, promoting more exploratory behaviors in potentially threatening environments.

3) MBT

As shown in Fig. 5, significant differences are found among the groups according to the results of the one-way ANOVA [$F(4,27) = 16.86$, $p < 0.0021$]. AW induced increased anxiety in

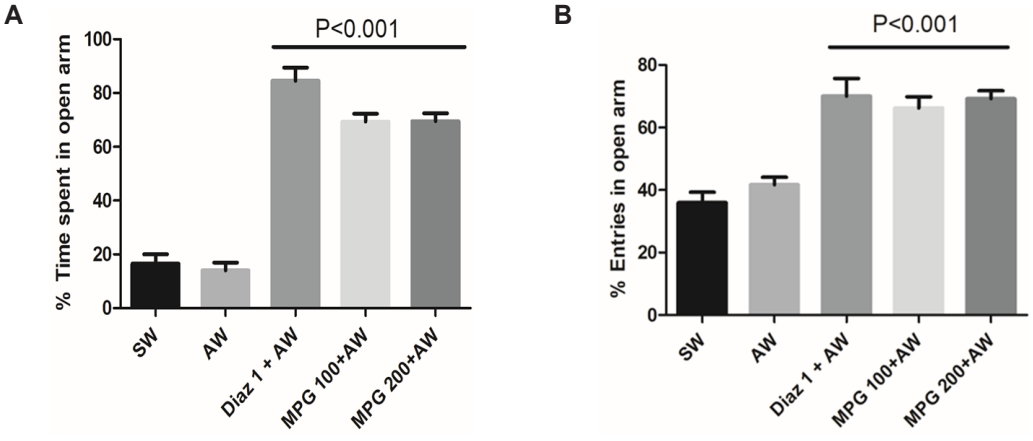

Figure 4. Effect of oral MPG (100 and 200 mg/kg) on (A) % open arm time spent, (B) % open arm entries in the elevated plus maze test in AW mice (N = 6-7).

 https://doi.org/10.3831/KPI.2024.27.3.234

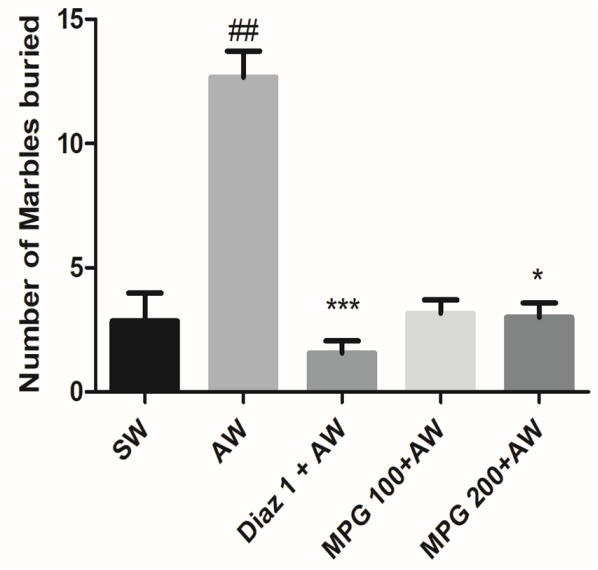

Figure 5. Effect of oral MPG (100 and 200 mg/kg) on total marbles buried in MBT in mice (N = 6-7). Statistical significance at *p < 0.05 and ***p < 0.001 when compared with the alcohol withdrawal control (AW) group; ##p < 0.01 when compared with the saline withdrawal control (SW) group.

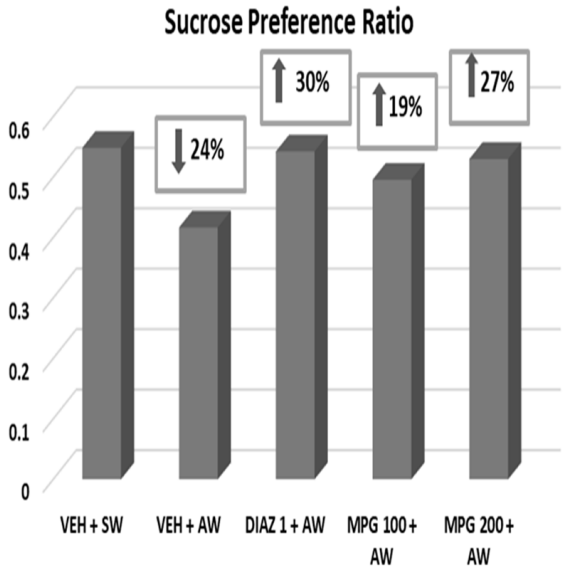

Figure 6. Effect of oral MPG (100 and 200 mg/kg) on sucrose preference ratio in mice SPT (N = 6-7).

mice, as evidenced by a greater number of marbles buried compared to the saline withdrawal mice. Notably, mice treated with diazepam showed a reduced tendency to bury marbles compared to those undergoing AW, confirming the anxiolytic effect of diazepam on the animals. Similarly, mice administered with MPG at oral doses of 100 mg/kg and 200 mg/kg also exhibited a significant reduction in marble-burying behaviors compared to AW mice. This finding demonstrated the anxiolytic-like effect of MPG in animals undergoing AW, suggesting its potential therapeutic benefit in alleviating anxiety-related behaviors in this context.

4. Evaluation of the anti-anhedonia effects of *Psidium guajava* leaves in alcohol withdrawal mice

1) SPT

In the SPT as shown in Fig. 6, significant differences were observed between the saline withdrawal and AW animals regarding sucrose intake. Specifically, AW animals exhibited a significant 24% reduction in sucrose consumption compared to the saline withdrawal mice, indicating reduced interest in experiencing pleasurable effects (anhedonia). Interestingly, oral administration of MPG at doses of 100 mg/kg and 200 mg/kg effectively normalized sucrose consumption in AW animals, similar to the effects observed with diazepam. These findings demonstrated the anti-anhedonic effect of MPG in AW mice, effectively restoring their preference for sucrose to levels comparable to those seen in control animals. These findings highlighted the potential therapeutic role of MPG in alleviating anhedonia associated with AW, suggesting its utility in promoting normal reward-seeking behaviors in this experimental model.

DISCUSSION

To prepare the ethanol solution used in our research, we employed 0.1% v/v fomepizole, an inhibitor of alcohol dehydrogenase that exhibits a significantly higher affinity for the enzyme compared to ethanol itself [11]. This inhibition effectively reduces the metabolism of ethanol, thereby increasing its bioavailability and prolonging its effects in experimental animals. This aids in studying the prolonged pleasurable effects of ethanol consumption.

Our study investigated the effects of MPG on anxiety and anhedonia induced by AW in mice. AW symptoms, such as anxiety, seizures, and depression, are intricately linked to neurotransmission dysfunction involving serotonin, GABA, and adrenergic receptors [12]. During AW, there is a reduction in GABA levels needed to maintain the balance between inhibitory and excitatory pathways in the brain. As ethanol is no longer available to enhance $GABA_A$ receptor function, this leads to

a decreased $GABA_A$ inhibitory neurotransmission, contributing to heightened anxiety and other symptoms [12].

Additionally, withdrawal symptoms may also result from increased activation of serotonin 5-HT2A receptors [13]. Studies have demonstrated that blocking 5-HT2C receptors with SB 242,084 exacerbates alcohol withdrawal symptoms in mice, while activating these receptors with mCPP alleviates them [14]. These findings highlight the involvement of 5-HT2C receptors in the development of AW symptoms. Chronic use of ethanol and its subsequent withdrawal are known to increase 5-HT2A receptor activity while decreasing activity in the benzodiazepine-$GABA_A$ergic inhibitory pathways. This imbalance contributes significantly to the depressive and anxiety-like effects observed during AW [12].

The bioactive components in the MPG, such as phenolic compounds (including guaijaverin, rutin, quercetin, kaempferol, and gallic acid), likely contribute to its observed effects. These compounds possess antioxidant properties and have been associated with various pharmacological activities, including neuropharmacological effects [15]. The findings of this study support the potential of MPG to mitigate anxiety and anhedonia associated with AW in mice. Future studies are warranted to identify the specific bioactive compounds responsible for these effects and elucidate their mechanisms of action. Furthermore, ongoing investigations are necessary to enhance our understanding of MPG, which may potentially lead to novel therapeutic interventions for AWS.

The MPG used in our study harnesses its pharmacological potential through a rich composition of phenolic compounds, including (+)-gallocatechin, quercetin, gallic acid, chlorogenic acid, procatechuic acid, caffeic acid, ferulic acid, and kaempferol. These compounds are renowned for their potent antioxidant properties and various neuropharmacological activities [15]. Methanol, due to its polarity, efficiently extracts these phenolic constituents from *Psidium guajava* leaves, as evidenced by the high phenolic content in MPG (5.271 mg/mL gallic acid equivalent), which surpasses extracts obtained from less polar solvents, such as butanol, ethyl acetate, and hexane [15]. Methanol's higher polarity compared to ethanol enhances its ability to extract these bioactive compounds, making it an optimal solvent for preparing MPG. In our research, we utilized 75% v/v methanol for the extraction of *Psidium guajava* leaves through maceration. This method ensures that the bioactive phenolic compounds responsible for the neuropharmacological effects of MPG are effectively extracted and retained in the extract.

Comparative studies have shown that aqueous organic solvents, such as 80% v/v methanol and 80% v/v ethanol, yield higher phenolic contents compared to absolute organic solvents in various medicinal plants. Specifically, aqueous methanol extracts have been reported to exhibit the highest extract yield and total phenolic content, often quantified in gallic acid equivalents [16]. This underscores the efficacy of aqueous methanol as an extraction solvent for obtaining phytoconstituents with potential therapeutic benefits.

In summary, using aqueous MPG in our study capitalizes on its high phenolic content and potent antioxidant properties, which are crucial for its protective effects against AW symptoms in mice. The choice of 75% v/v methanol for extraction ensures the retention of these bioactive compounds, facilitating the exploration of MPG's therapeutic potential in managing alcohol-related disorders.

The current research highlights the potential of MPG in alleviating anxiety and anhedonia in AW mice. This extract has demonstrated anxiolytic-like effects in behavioral tests including OFT and EPM, which are standard models for assessing anxiety-related behaviors in rodents. Similar anxiolytic effects were previously reported with the ethanolic extract of *Psidium guajava* Linn leaves (EEPG) in mice [17], indicating consistent behavioral benefits across different solvent extracts of guava leaves.

Phytochemical studies have identified several bioactive compounds in guava leaves, including guaijaverin, rutin, quercetin, kaempferol, naringenin, epicatechins, catechins, and gallic acid [18]. Among these, rutin, quercetin, and kaempferol have been specifically noted for their anxiolytic activities in previous studies, primarily through modulation of GABAergic neurotransmission [19-21]. The GABAergic system is crucial for maintaining the balance between inhibitory and excitatory neurotransmission in the brain, and its dysregulation is implicated in anxiety disorders and AW symptoms.

Based on these findings, it is hypothesized that the anxiolytic effects of MPG observed in this study are mediated, at least in part, by its influence on GABAergic processes. By enhancing GABAergic neurotransmission, MPG may mitigate the anxiety and anhedonia associated with AW in mice.

Further research is warranted to isolate and identify the specific bioactive compounds responsible for these effects and to elucidate their mechanisms of action in AW models. In conclusion, the MPG shows promise as a natural therapeutic agent for managing anxiety and anhedonia induced by AW, potentially

through its modulation of GABAergic pathways. Continued investigation into the bioactive components of MPG and their pharmacological actions will be crucial for developing novel treatments for alcohol-related disorders.

CONCLUSION

The findings of this study demonstrated the potential antidepressant-like and anxiolytic-like effects of *Psidium guajava* leaf methanolic extract (MPG) in mitigating AW-induced symptoms in mice. The key findings and implications of this study were as follows.

1. Antidepressant-like activity: MPG normalized sucrose intake in AW mice at oral dosages of 100 mg/kg and 200 mg/kg. Anhedonia, characterized by reduced pleasure-seeking behaviors (such as reduced sucrose intake), is a common symptom of AW. The ability of MPG to restore sucrose preference suggests potential antidepressant-like effects.

2. Anxiolytic-like effects: Oral administration of MPG at doses of 100 mg/kg and 200 mg/kg increased the time spent in the central area during the OFT and in the open arms of the EPM among the AW mice. These behaviors indicate reduced anxiety-like responses in challenging environments. MPG reduced the number of marbles buried by AW mice in the MBT. This test aims to assess for repetitive and anxiety-related behaviors in rodents. A reduction in marble-burying indicates decreased anxiety.

3. Mechanism of action: MPG may provide protective effects against AW symptoms, including anxiety and anhedonia, by modulating $GABA_A$-ergic mechanisms. The GABAergic system plays a critical role in regulating anxiety and mood disorders, and enhancing GABAergic neurotransmission could potentially alleviate AW symptoms.

4. Phytochemicals: Flavonoid compounds found in *Psidium guajava* leaves, such as rutin, quercetin, and kaempferol, are potentially therapeutic in treating AW symptoms. These compounds have been previously associated with GABA-$_A$ergic-mediated effects and may have contributed to the observed behavioral effects of MPG.

5. Future directions: Ongoing laboratory research studies are warranted to identify and characterize the specific phytochemicals responsible for MPG's therapeutic effects against AW. Further preclinical studies are also needed to elucidate the exact mechanisms of action and explore the clinical relevance of MPG in humans.

6. Clinical implications: The findings contribute to the growing body of evidence supporting the use of natural compounds, such as MPG, for managing AW symptoms. If the therapeutic effects of MPG are validated in human studies, MPG could offer a novel therapeutic approach for individuals experiencing anxiety and anhedonia during AW.

In conclusion, the present study suggests that MPG is a promising natural remedy for alleviating AW-associated anxiety and anhedonia, potentially through its effects on $GABA_A$-ergic neurotransmission. Continued research is essential to fully understand its mechanisms and to translate these findings into clinical applications.

ACKNOWLEDGEMENTS

We express our sincere gratitude to DST-SERB for the research grant (No. CRG/2018/000813) for carrying out this project. We are thankful to Prof. Rama Rao N, the Principal, Chalapathi Institute of Pharmaceutical Sciences, and Sri YV Anjaneyulu, the president, Chalapathi Educational Society, Lam, Guntur for providing the necessary lab facilities to perform this study.

AUTHORS' CONTRIBUTIONS

VP conceived, designed, analyzed, drafted, and critically revised the manuscript; VH conducted the experiments, interpreted and analyzed the data, and wrote the manuscript. Both authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

FUNDING

This research project was funded by DST-SERB, India (Research grant No. CRG/2018/000813).

ORCID

Vanjarapu Harithadevi, https://orcid.org/0009-0004-8031-4129
Vijayapandi Pandy, https://orcid.org/0000-0002-0524-4800

REFERENCES

1. Pandy V, Challa H, Byram P. Protective effect of methanolic extract of Areca catechu nut on ethanol withdrawal symptoms in mice. Futur J Pharm Sci. 2023;9:10.
2. World Health Organization (WHO). Alcohol [Internet]. WHO [cited 2023 Oct 30]. Available from: https://www.who.int/news-room/fact-sheets/detail/alcohol
3. Mirijello A, D'Angelo C, Ferrulli A, Vassallo G, Antonelli M, Caputo F, et al. Identification and management of alcohol withdrawal syndrome. Drugs. 2015;75(4):353-65.
4. Gupta A, Khan H, Kaur A, Singh TG. Novel targets explored in the treatment of alcohol withdrawal syndrome. CNS Neurol Disord Drug Targets. 2021;20(2):158-73.
5. Kumar M, Tomar M, Amarowicz R, Saurabh V, Nair MS, Maheshwari C, et al. Guava (*Psidium guajava* L.) leaves: nutritional composition, phytochemical profile, and health-promoting bioactivities. Foods. 2021;10(4):752.
6. Devi VH, Dukkipati S, Pandy V. A review on phytochemical, biological, and pharma-cological effects of *Psidium guajava Linn*. (Guava). Int J Pharm Sci. Nanotech. 2024;17(1):7173-82.
7. Arya V, Thakur N, Kashyap CP. Preliminary phytochemical analysis of the extracts of *Psidium* leaves. J Pharmacogn Phytochem. 2012;1(1):1-5.
8. Seibenhener ML, Wooten MC. Use of the Open Field Maze to measure locomotor and anxiety-like behavior in mice. J Vis Exp. 2015;96:e52434.
9. Narasingam M, Vijeepallam K, Mohamed Z, Pandy V. Anxiolytic- and antidepressant-like activities of a methanolic extract of Morinda citrifolia Linn. (noni) fruit in mice: involvement of benzodiazepine-$GABA_A$ergic, serotonergic and adrenergic systems. Biomed Pharmacother. 2017;96:944-52.
10. Kedia S, Chattarji S. Marble burying as a test of the delayed anxiogenic effects of acute immobilisation stress in mice. J Neurosci Methods. 2014;233:150-4.
11. Farook JM, Krazem A, Lewis B, Morrell DJ, Littleton JM, Barron S. Acamprosate attenuates the handling induced convulsions during alcohol withdrawal in Swiss Webster mice. Physiol Behav. 2008;95(1-2):267-70.
12. Becker HC, Mulholland PJ. Neurochemical mechanisms of alcohol withdrawal. Handb Clin Neurol. 2014;125:133-56.
13. Lovinger DM. Serotonin's role in alcohol's effects on the brain. Alcohol Health Res World. 1997;21(2):114-20.
14. Fu R, Mei Q, Shiwalkar N, Zuo W, Zhang H, Gregor D, et al. Anxiety during alcohol withdrawal involves 5-HT2C receptors and M-channels in the lateral habenula. Neuropharmacology. 2020;163:107863.
15. Tachakittirungrod S, Okonogi S, Chowwanapoonpohn S. Study on antioxidant activity of certain plants in Thailand: mechanism of antioxidant action of guava leaf extract. Food Chem. 2007;103(2):381-8.
16. Sultana B, Anwar F, Ashraf M. Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. Molecules. 2009;14(6):2167-80.
17. Biswas S, Mondol D, Jodder P, Sana S, Saleh MA, Tarafdar AK, et al. Evaluation of neurobehavioral activities of ethanolic extract of *Psidium guajava* Linn leaves in mice model. Futur J Pharm Sci. 2021;7(1):36.
18. Ruksiriwanich W, Khantham C, Muangsanguan A, Phimolsiripol Y, Barba FJ, Sringarm K, et al. Guava (*Psidium guajava* L.) leaf extract as bioactive substances for anti-androgen and antioxidant activities. Plants (Basel). 2022;11(24):3514.
19. Hernandez-Leon A, González-Trujano ME, Fernández-Guasti A. The anxiolytic-like effect of rutin in rats involves GABAA receptors in the basolateral amygdala. Behav Pharmacol. 2017;28(4):303-12.
20. Islam MS, Hossain R, Ahmed T, Rahaman MM, Al-Khafaji K, Khan RA, et al. Anxiolytic-like effect of quercetin possibly through GABA receptor interaction pathway: in vivo and in silico studies. Molecules. 2022;27(21):7149.
21. Grundmann O, Nakajima J, Kamata K, Seo S, Butterweck V. Kaempferol from the leaves of Apocynum venetum possesses anxiolytic activities in the elevated plus maze test in mice. Phytomedicine. 2009;16(4):295-302.