**Therapeutic potential of *Anacyclus pyrethrum* aqueous extract in managing Clonazepam withdrawal in rats**

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**ABSTRACT**

The management of benzodiazepine withdrawal syndrome is challenging, and there is currently no consensus on the optimal treatment approach. Our study aims to investigate the possible effect of the aqueous extract of *Anacyclus pyrethrum* (AEAP) on Clonazepam dependence in rats, via the measurement of oxidative stress, behavioral and biochemical changes. Dependence was induced by chronic administration of Clonazepam for 30 days, then AEAP was administered orally to rats. Withdrawal syndrome was assessed employing the conditioned place preference test, behavioral tests, and biochemical assays. Oxidative stress was evaluated by measuring levels of malondialdehyde (MDA), catalase (CAT), and superoxide dismutase (SOD). The percentage of open-arm entries, time spent in open arms decreased, and immobility time increased significantly among the Clonazepam-dependent group from the preconditioning to withdrawal phase \(p < 0.001\). The cortisol level was higher among Clonazepam group, the MDA increased, and CAT and SOD measurements demonstrated a notable decrease in the Clonazepam withdrawal group. AEAP administration at a dose of 200 mg/kg significantly alleviated anxiety and depression-like behaviors and led to a substantial decrease in oxidative stress levels during Clonazepam withdrawal, demonstrating the beneficial impact of AEAP. The use of *Anacyclus pyrethrum* may provide a novel therapeutic approach for managing the adverse effects of Clonazepam dependence by targeting oxidative stress.

**INTRODUCTION**

Substance use disorders (SUDs) pose a considerable public health problem, with an estimated 284 million individuals worldwide reported to have experimented with drugs in 2020 [1] despite evidence highlighting the negative influence of substance abuse on both individuals and the broader society [2]. Illicit drug use remains widespread, contributing significantly to the total load of diseases. Still, the underlying factors that contribute to vulnerability to dependence are still completely noncomprehended, and there is a lack of effective treatments available [3].

Benzodiazepines such as Clonazepam are considered the most used psychotropic medications in psychiatry for treating different disorders such as epilepsy, depression, anxiety, and sleep disorders [4,5]. However, benzodiazepines are also highly addictive and can lead to dependence, withdrawal symptoms, and other adverse effects [6]. Despite these risks, benzodiazepines continue to be widely prescribed, and their misuse remains a major public health concern.

Clonazepam addiction is a major health problem; around 20%-30% of people become addicted after 1 month of use [3,7]. The withdrawal syndrome associated with Clonazepam dependence can be severe and may include symptoms such as seizures, anxiety, insomnia, and tremors [8]. The management of this withdrawal syndrome is difficult, and there is currently no consensus on the optimal therapeutic approach [9].

Persistent utilization of benzodiazepines, including Clonazepam, has been consistently linked to modifications...
within several neurotransmitter systems encompassing
gamma-aminobutyric acid (GABA), serotonin, and dopamine,
ultimately exerting profound repercussions on the structure
and functioning of the brain. Noteworthy, the disrupted equilibrium
of these neurotransmitters has been implicated in the observed
alterations occurring in key brain regions that regulate emotional
responses and cognitive processes, notably the prefrontal cortex,
hippocampus, and amygdala. These regions play pivotal roles
in emotion regulation, memory consolidation, and decision-
making, and any disturbances in neurotransmitter function can
substantially impact their normal functioning [9,10]. In addition
to these neurotransmitter systems, emerging research suggests
that the role of glutamate receptors should be considered in
understanding the effects of benzodiazepine addiction on the
brain. Glutamate is a primary excitatory neurotransmitter in the
central nervous system (CNS) that plays a critical role in synaptic
plasticity, learning, and memory. Dysregulation of glutamate
receptors, particularly the N-methyl-D-aspartate (NMDA)
receptor subtype, has been implicated in the pathophysiology

The oxidative stress associated with benzodiazepine
dependence and Clonazepam use may further impact glutamate
receptor function and contribute to neuronal damage and
dysfunction [12]. Oxidative stress, characterized by an
imbalance between the production of reactive oxygen species
(ROS) and the antioxidant defense systems, has been linked
to anxiety and depressive disorders frequently observed in
individuals with benzodiazepine dependence [13]. The interplay
between oxidative stress and glutamate receptors suggests a
potential mechanistic pathway that underlies the adverse effects
of Clonazepam addiction on brain function. Oxidative stress-
induced modifications of glutamate receptors, particularly
NMDA receptors, can lead to dysregulated glutamatergic
neurotransmission, excitotoxicity, and neuronal damage. These
changes may contribute to the development of withdrawal
symptoms and exacerbate the overall impact of Clonazepam
dependence on brain function [14].

Hence, continued research of novel features to prevent,
predict, and treat the stages of drug abuse and dependence are
necessary. Since an important rate of relapse is noticed among
patients suffering from SUD, even after prolonged abstinence,
it is a crucial challenge to expand potent therapeutic procedures
to control SUD affecting the brain and to attenuate withdrawal
syndrome [15].

The utilization of natural products, including
drugs used in recent studies, as a means of obtaining therapeutic agents
is experiencing a surge in popularity. This is primarily due to their
potential effectiveness and comparatively lower toxicity when
compared to synthetic drugs. Natural products are increasingly
recognized as promising alternatives for treating and/or
preventing neuropsychiatric conditions such as SUD [15].

Anacyclus pyrethrum (A. pyrethrum) is a plant
belonging to the Asteraceae family and to the Anacyclus genus.
It is commonly known as pellitory, Aqar Qarha, Oud El Attas,
and tigandizt. Anacyclus pyrethrum is a medicinal plant that has
been used for various ailments, including pain, inflammation,
and fever. However, its potential utility as a therapeutic agent
for managing the adverse effects of Clonazepam dependence
has not been investigated. Given the association between
Clonazepam dependence and oxidative stress, it is possible that
targeting oxidative stress may represent a novel therapeutic
approach for managing the adverse effects of Clonazepam
dependence. Therefore, the proposed study could contribute
to a good comprehension of the role of oxidative stress in
the adverse effects of Clonazepam dependence, as well as
the potential utility of A. pyrethrum as a therapeutic agent for
managing these effects.

MATERIAL AND METHODS

Plant material

The collection of A. pyrethrum roots took place in
Bin El Ouidan, located near Marrakech, Morocco (32° 7'48" 
latitude N/6° 27'36" longitude W). The roots were first verified
for authenticity by Pr. Chait and then stored under the MARK-
1003 voucher in the plant herbarium of the Faculty of Sciences
Semlalia, Marrakech. The extraction process of the plant
involved the use of distilled water, with minor adjustments
made to a previously described method [16]. After 24 hours of
stirring, the aqueous extract was first filtered, then the powder
form was obtained after lyophilization, and stored at 4°C. The
final yield of this extraction was 17.1%.

During this investigation, the toxicity of the aqueous
extract of Anacyclus pyrethrum (AEAP) was assessed using
doses of 1,000, 2,000, and 5,000 mg/kg. The results indicated
that these doses were safe, as there were no instances of
mortality nor alterations in body and organ weights after a 14-
day administration period. Furthermore, the LD50 value of
the AEAP was determined to be >5,000 mg/kg, showing the
extract's nontoxicity [17].

Animals

Male Sprague-Dawley rats, weighing 190 ± 15 g, were
housed in transparent cages in a controlled environment.
The temperature was maintained at 22°C ± 2°C, while the humidity
was kept at 50% ± 10%. A 12:12-hour light/dark cycle was
followed. The rats had unrestricted access to both water and food,
which were provided ad libitum. Before the commencement
of the experiments, the rats were acclimatized to the laboratory
environment for a period of 7 days. All procedures involving
animals were carried out in compliance with the guidelines
outlined by the European Council Directive for Care and Use
of Laboratory Animals (EU2010/63). Before the commencement,
the Institutional Local Review Board approved the study
protocol for animal experimentation, with the protocol code
CA965/07/22 and an approval date of October 2022.

Drugs administration for physical dependence

The Clonazepam used in this study was commercially
available. It was administered intraperitoneally (i.p) in an
escalating dose regimen, starting with 1.5 mg/kg/day and
gradually increasing by 10% each day, up to a maximum dose
of 6 mg/kg/day. The Clonazepam was solubilized in a saline
solution and administered daily for a period of 30 days. This
dosing protocol aimed to mimic the progressive increases in
Benzodiazepine consumption observed in human addiction, as supported by previous research studies [5,18].

**Treatment and grouping**

Following the acclimatization period to the laboratory environment, the animals were then assigned into four different groups, with each group consisting of six animals:

1. Vehicle (control; saline solution 0.9%).
2. Clonazepam dependent group: 30 days of daily administration of Clonazepam followed by a withdrawal phase (07 days).
3. AEAP treatment group (200 mg/kg; orally), the choice of that dose was based on our prior studies [19–21].
4. Clonazepam + AEAP group; Clonazepam-dependent treated group after 30 days of daily administration of Clonazepam followed with AEAP treatment (200 mg/kg; orally) for 1 week from day 34 to 40.

**Conditioned place preference (CPP)**

The Clonazepam-induced CPP test was conducted following a previously established protocol [19,22]. The CPP apparatus consisted of three Polyvinyl chloride compartments: two larger conditioning side chambers (30 × 25 × 30 cm) with distinct somatosensory cues such as colored walls (white or zebra) and different floor surfaces and a middle neutral chamber (11 × 25 × 30 cm). The CPP procedure consisted of three phases: preconditioning, conditioning, and postconditioning (dependence). During the preconditioning (days 1–3), rats were confined to the white compartment with open doors, allowing them unrestricted access to both compartments. Their behavior and preferences were observed and recorded for 35 minutes to establish the baseline preference for each chamber. Rats showing a clear preference for one side compartment over the others were excluded from the study. In the second phase (conditioning: days 4–9), rats received alternating injections of either Clonazepam or saline solution two times per day: in the morning (10:00 a.m), and evening (8:00 p.m.) for 6 days. After receiving Clonazepam, the rats were confined to the zebra compartment for 45 minutes, while after saline injection, they were confined to the white compartment. The control group received saline injections during the rotated sessions throughout the conditioning and postconditioning phases. In the postconditioning phase (days 10–33), the rats underwent re-testing for Clonazepam-CPP. They were given free access to both the white and zebra chambers for 15 minutes, and their behavior was monitored using a camera connected to a computer interface. The number of entries to the Clonazepam-paired chamber and the total entries were recorded to calculate the CPP score. The time spent in each compartment was also measured. At the end of the withdrawal phase (day 40), the rats were allowed unrestricted entry to all apparatus, and their behavior was observed for 15 minutes.

**Behavioral tests**

On days 1 and 40, behavioral tests were carried out between 09:00 and 15:00 in a soundproof room with the experimental rats.
density lipoprotein (LDL), high-density lipoprotein (HDL), and cortisol. The biochemical analyses were performed using a standard technique with a Cobas 6,000 machine from Roche.

Blood collection and preparation of brain tissue samples

After 40 days, the animals were sacrificed by decapitation, and the blood was drawn for biochemical analyses. Later, the rats’ brains were immediately removed and cooled on dry ice. Then, the hippocampus was dissected on ice, with reference to a rat cerebral atlas in order to carry out an enzymatic assay of oxidative stress [26]. The hippocampus was chosen since it is considered the most important brain region in relation to oxidative stress and neurodegeneration associated with Clonazepam dependence; the hippocampus is highly sensitive to oxidative stress-induced damage [27,28].

Oxidative stress assessment

The hippocampus tissues were homogenized in 20-mM Tris-HCl buffer (pH 7.4) on ice, and were subsequently used to determine the levels of lipid peroxidation (LPO), the activities of catalase (CAT) and superoxide dismutase (SOD).

LPO assay

The level of LPO was assessed by measuring the thiobarbituric acid-reacting substances (TBARSs) in the hippocampus homogenates, following a previous protocol [29]. In brief, a portion of the crude homogenate from the hippocampus weighing 100 mg was subjected to centrifugation at 4°C (1,000 × g for 10 minutes). The resulting supernatant was then combined with 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid. The mixture was heated in a boiling water bath for 15 minutes, followed by the addition of butanol (2:1, v/v) to the solution. After another round of centrifugation (800 × g for 05 minutes), the absorbance at 535 nm was measured to determine the concentration of TBARS. The outcomes were expressed as nanomoles of malondialdehyde (MDA) per gram of wet tissue.

CAT activity

CAT activity was determined using an H$_2$O$_2$-dependent method to measure the production of H$_2$O and O$_2$ [30]. In brief, 0.05 ml of the sample was mixed with 1 ml of H$_2$O$_2$ solution (0.019 M) and 1.95 ml of 50-mM phosphate buffer in a 3-ml quartz cuvette. The absorbance of the mixture was measured at 240 nm at time 0 (T0) and then at 30-second intervals for 2 minutes.

SOD activity

SOD activity was determined by measuring its ability to inhibit the photoreduction of nitro blue tetrazolium (NBT) using spectrophotometric methods [31]. The assay systems were prepared by combining 2.4 × 10$^{-6}$ M riboflavin, 0.01 M methionine, 1.67 × 10$^{-4}$ M NBT, and 0.05 M potassium phosphate buffer at pH 7.4 and 25°C. This reaction mixture, under aerobic conditions, resulted in a blue color, and its optical density was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme protein that caused a 50% reduction in the rate of NBT reduction.

Statistical analysis

Statistical analysis of the data was conducted using GraphPad Prism Software version 9.00 (San Diego, California, USA). The results are presented as the mean ± SEM. One-way analysis of variance (ANOVA) was performed, followed by post-hoc Tukey’s tests, to assess the differences between groups. A significance level of $p < 0.05$ was considered statistically significant in determining the observed group differences.

RESULTS

Clonazepam-induced CPP

Clonazepam preference was measured using the CPP test, where rats were administered a daily escalating dose of Clonazepam, starting at 1.5 mg/kg/day during the initial phase. No significant differences were observed in the time spent and percentage of entries to the Clonazepam-paired chamber among the groups (Fig. 1A and B). However, the daily administration of Clonazepam significantly increased the time spent and percentage of entries to the Clonazepam-paired chamber compared to the vehicle group ($p < 0.01$) and the initial phase ($p < 0.01$), indicating the establishment of CPP for Clonazepam. The rats spent 235 seconds in the Clonazepam-compartment. In the Clonazepam-dependent withdrawal group treated with AEAP, a decrease was noted in the time spent (121 seconds) and percentage of entries to the drug-paired chamber compared to the Clonazepam-dependent rats (Fig. 1A and B).

Effects of AEAP on rats withdrawn from Clonazepam on behavioral parameters

To evaluate withdrawal symptoms, anxiety-like and depression-like behaviors were assessed by measuring the time spent and the percentage of entries to the open arms in the EPM test (Fig. 2). During the preconditioning phase, the results showed that there were no significant differences across the groups for EPM and % of entries to the open arms. However, during the withdrawal phase, the Clonazepam withdrawn group exhibited a significant decrease in both the time spent and the percentage of entries to the open arms compared to the vehicle group ($p < 0.001$) and the preconditioning phase ($p < 0.001$), indicating the presence of anxiety-like behavior during withdrawal (Fig. 2A and B). On the other hand, both parameters increased in the Clonazepam withdrawn group treated with AEAP versus Clonazepam withdrawn group ($p < 0.001$), expressing the anxiolytic effect of AEAP (Fig. 2A and B). As illustrated in Figure 3, a decrease in the number of rearing (Fig. 3A), and number of crossed lines (Fig. 3B) were significantly noticed in Clonazepam-induced rats during the withdrawal phase than vehicles ($p < 0.001$) and initial phase ($p < 0.001$), and those traits were mitigated in the Clonazepam-exposed rats treated with AEAP compared to the Clonazepam withdrawn group ($p < 0.001$). Rats in the AEAP group yielded a significant increase in the number of rearing (Fig. 3A) and the number of lines crossed (Fig. 3B) in the OFT as compared to the normal control rats. Significant differences in the immobility time were observed among the various groups in the FST, which is commonly used to assess depression-like behavior (Fig. 3C), according to ANOVA. Data showed a significant increase in
Effects of AEAP on rats withdrawn from Clonazepam on oxidative stress markers

Figure 5A illustrates the MDA measurement as a means of evaluating LPO due to oxidative stress. The results demonstrated a notable increase in the Clonazepam withdrawal group (35.21 ± 2.43) compared to the vehicle group (11.01 ± 0.30). However, post-treatment with AEAP in the Clonazepam withdrawal group produced a significant reduction in MDA levels ($p < 0.001$; Fig. 5A), suggesting a positive effect on oxidative stress. In addition, CAT and SOD levels significantly decreased in the Clonazepam withdrawal group compared to the vehicle group ($p < 0.001$). However, post-treatment with AEAP in the Clonazepam withdrawal rats significantly increased CAT and SOD levels ($p < 0.001$; Fig. 5B and C), further elucidating the beneficial effect of AEAP on oxidative stress.

DISCUSSION

This study aimed to investigate behavioral, biochemical, and oxidative stress response to chronic administration of Clonazepam, and treatment with the AEAP in rats. The current study exhibited that oral administration of $A. pyrethrum$ attenuated withdrawal syndrome in Clonazepam-dependent rats, as shown by immobility time in Clonazepam withdrawn rats ($p < 0.001$) than in control rats and the preconditioning phase. However, treatment with AEAP for the Clonazepam-withdrawn group decreased the immobility time as compared to the Clonazepam withdrawn group ($p < 0.001$), confirming the anti-depressant effect.

Effects of AEAP on rats withdrawn from Clonazepam on biochemical markers

As the withdrawal phase induces stress, cortisol levels were measured as a biochemical marker to assess the stress response. The Clonazepam group showed higher cortisol levels compared to the vehicle group, with values of 13.54 (g/L) ± 1.68 and 6.4 (g/L) ± 2.23, respectively, indicating a significant difference ($p < 0.001$; Fig. 4F). However, in the Clonazepam-dependent group treated with AEAP, the cortisol level significantly decreased compared to the Clonazepam-dependent group ($p < 0.01$; Fig. 4F). In addition, CRP, a marker of inflammation produced by the liver, showed a significant increase in the Clonazepam-dependent group compared to the vehicle group ($p < 0.001$). However, post-treatment with AEAP reduced CRP levels ($p < 0.001$; Fig. 4E). On the other hand, there were no significant differences in the levels of other biochemical markers, including total cholesterol, triglycerides, HDL, and LDL, among all groups ($p > 0.05$; Fig. 4A–D).
decreased preference in the Clonazepam-paired chamber in the CPP test, decreased immobility time in the FST, increased locomotor activity, rearing in the OFT compared to Clonazepam-dependent rats and mitigated Clonazepam-induced oxidative stress.
Effects of withdrawal phase. Statistical analyses were done according to one-way ANOVA, Clonazepam on oxidative stress: (A) MDA, (B) CAT, (C) SOD during the withdrawal phase. Statistical analyses were done according to one-way ANOVA, followed by Tukey post hoc test, *** p < 0.0001, ** p < 0.01. ns p > 0.5.

Figure 5. Effects of A. pyrethrum aqueous extract on rats withdrawn from Clonazepam on oxidative stress: (A) MDA, (B) CAT, (C) SOD during the withdrawal phase. Statistical analyses were done according to one-way ANOVA, followed by Tukey post hoc test, *** p < 0.0001, ** p < 0.01. ns p > 0.5.

In this study, CPP was utilized to evaluate preference-like behavior in rats. Through this paradigm, the rats learned to associate a specific chamber with Clonazepam administration and, during withdrawal, displayed a preference for the Clonazepam-paired chamber. This current research provides insight into the incentive-motivational value of Clonazepam for dependent rats, particularly when Clonazepam is abruptly stopped after 30 days of continuous administration with an escalating dose regimen designed to mimic the drug consumption pattern observed in humans. Similarly, another study found that rats exhibited a preference for a Clonazepam-associated environment during the acute withdrawal phase, suggesting that the rewarding effects of Clonazepam may contribute to the development of dependence [32]. The resulting brain concentrations were comparable to those that would be expected in humans receiving usual therapeutic doses [33]. The chronic administration of Clonazepam leads to tolerance related to changes in the density and function of GABA type A receptors in the brain. Specifically, chronic activation of these receptors by benzodiazepines leads to a downregulation of receptor density, resulting in a decreased sensitivity to the drug. This downregulation occurs after approximately 7 days of continuous benzodiazepine use and can be observed in various regions of the brain, including the hippocampus, amygdala, and cortex. The downregulation of GABA type A receptors is thought to be a compensatory response to the chronic presence of benzodiazepines, and it can contribute to the development of tolerance and dependence [3,34].

Furthermore, in the current study, increased stress sensitization was observed following Clonazepam dependence. This was assessed by measuring cortisol levels in the blood as an indicator of anxiety-like behavior, which is known to be mediated by the corticotrophin-releasing factor (CRF) system. Chronic administration of benzodiazepines has been shown to affect the functioning of the CRF system, which plays a key role in the regulation of the stress response. Specifically, benzodiazepine use has been associated with changes in the density and function of CRF receptors, as well as alterations in gene expression [34]. These changes can contribute to the development of anxiety and stress-related disorders and may also contribute to the withdrawal syndrome that occurs following benzodiazepine cessation [35].

The current study revealed behavioral impairments in the Clonazepam-dependent group, which included a decrease in the number of crossed lines and rearing in open-field activity. These findings are consistent with previous research that has reported reduced exploratory activity and hypolocomotion in rodents following chronic benzodiazepine administration [36,37]. In addition, the current study demonstrated an increase in stress sensitization in the Clonazepam-dependent rats, as evidenced by elevated cortisol levels in the blood. This finding is consistent with previous research that has linked CRF to anxiety-like behavior during drug withdrawal [38]. Specifically, CRF has been identified as a crucial factor in the manifestation of anxiety-like symptoms during alcohol and benzodiazepine withdrawal. Studies have demonstrated that CRF levels increase in response to stress and are associated with anxiety, depression, and other mood disorders. In the context of substance withdrawal, the release of CRF in the brain has been linked to the onset of withdrawal symptoms such as anxiety, agitation, and dysphoria [39,40].

The current study revealed that daily administration of Clonazepam caused an increase in depressive-like behavior during the withdrawal phase. Prolonged activation of GABAergic receptors by Clonazepam leads to a decrease in serotonin levels; this reduction in serotonin availability subsequently influences neural plasticity and structural modifications, along with CNS hyperexcitability. In the CNS, inhibitory synapses play a crucial role in regulating the number of principal neurons, thereby impacting overall CNS functioning. Moreover, the activation of μ-opioid receptors on GABAergic interneurons within the ventral tegmental area plays a significant role in this context. When these μ-opioid receptors are activated, they inhibit GABAergic interneurons, leading to disinhibition of dopaminergic neurons. Consequently, the release of dopamine in the nucleus accumbens is heightened. These interconnected mechanisms contribute to the complex effects of Clonazepam use on neural function and the development of addictive behaviors [6,41,42].

Oxidative stress is known to play a critical role in various pathological conditions, including drug dependence and withdrawal. In this study, we found that Clonazepam withdrawal resulted in a significant increase in LPO, as evidenced by elevated MDA levels. This finding is consistent with previous studies that have shown a correlation between LPO and drug withdrawal syndrome [43,44]. The observed decrease in CAT and SOD levels further supports the involvement of oxidative stress in Clonazepam withdrawal [45,46]. During chronic exposure to
Clonazepam, the continuous activation of GABA receptors leads to a decrease in serotonin levels, which in turn causes a decrease in the activity of antioxidant enzymes [47,48]. This imbalance leads to an accumulation of ROS and oxidative damage to cellular structures, including lipids, proteins, and DNA [49]. Furthermore, dopamine, as a prominent neurotransmitter that is involved in reward and addiction processes, has also been implicated in the interplay between oxidative stress and Clonazepam withdrawal. Dopamine metabolism can generate ROS as byproducts, thereby contributing to oxidative stress [50–52].

Medicinal plants have been extensively used due to their antioxidant potential, safe for long-term use, and being considered an alternative treatment option due to their availability, low cost, and lack of side effects [53]. Anacyclus pyrethrum is a medicinal plant commonly used in traditional medicine to treat various ailments such as rheumatism, toothache, and dyspepsia [54]. It is known to possess several bioactive compounds, including flavonoids, phenols, and terpenoids, which exhibit potent antioxidant and anti-inflammatory activities.

In our preliminary investigation, we conducted a systematic evaluation of AEAP’s pharmacological effects using various doses (100, 200, 400, and 800 mg/kg). Notably, our initial findings highlighted the 200 mg/kg dose as particularly effective in producing therapeutic effects. This pivotal outcome informed our deliberate choice of the 200 mg/kg dose for further exploration in our study [19]. Before addressing the effects of A. pyrethrum on withdrawal, it is crucial to highlight that the current study demonstrated significant anxiolytic and antidepressant activity of the AEAP. In FST, AEAP reduced immobility times compared to the control group, indicating strong antidepressant activity. Similar findings have been reported [55–57], indicating that the ethanolic extract of A. pyrethrum also acts as an antidepressant in mice. The observed decrease in mortality is comparable to the effects observed with a reference antidepressant, further supporting the consistency of our results with other studies. In addition, it was proposed that the root extract of A. pyrethrum may exert an antidepressant effect by interacting with the adrenergic or dopaminergic system, resulting in elevated levels of norepinephrine and dopamine [57].

Moreover, in behavioral tests such as the OFT and the EPM, AEAP displayed anxiolytic potential by increasing exploratory and locomotor activities, as evidenced by elevated central crossed lines, peripheral crossed lines, and rearing behavior in the OFT, as well as increased exploration of open arms and higher open arm entries in the EPM. These observed anxiolytic effects are consistent with the results from another study [58] investigating the effects of the ethanol extract of A. pyrethrum in mice, which revealed an increase in the time spent in the light compartment and alterations in the number of shuttle crossings, indicating its anxiolytic activity. The anxiolytic effects observed may be attributed to the compound’s agonistic effects on the GABA/benzodiazepine receptor complex, the 5-HT1A receptor, or its ability to antagonize the 5-HT1B receptor [59,60].

Furthermore, the potential therapeutic value of A. pyrethrum for managing anxiety and depression can be attributed to the presence of bioactive compounds, particularly alkylamides. These compounds are known to increase the level of GABA in the brain, leading to reduced anxiety and promotion of relaxation [19].

Moving on to the effects of A. pyrethrum during Clonazepam withdrawal, our study revealed promising outcomes in mitigating withdrawal syndrome, akin to the observed anxiolytic, antidepressant effects, as well as cortisol and oxidative stress reduction. Upon discontinuation of Clonazepam, animals typically exhibit withdrawal symptoms, which include heightened anxiety, depression, and increased cortisol levels indicative of stress response. However, AEAP treatment displayed a noteworthy alleviation of withdrawal symptoms.

Moreover, in light of the dysregulation of dopamine homeostasis observed during Clonazepam withdrawal, the noteworthy alleviation of withdrawal symptoms demonstrated by treatment with AEAP highlights the potential interplay between neurotransmitter function, oxidative stress, and the observed beneficial effects during Clonazepam withdrawal. The dysregulation of both serotonin and dopamine systems during Clonazepam withdrawal likely contributes to the generation of oxidative stress, further exacerbating cellular damage. This complex relationship between neurotransmitters, GABA receptors, oxidative stress, and the observed alleviation of withdrawal symptoms underscores the potential therapeutic value of A. pyrethrum in managing Clonazepam withdrawal [61,62].

Anacyclus pyrethrum has been shown to have a protective effect against several neurological disorders, such as Alzheimer’s and Parkinson’s diseases; these disorders are also associated with oxidative stress. The neuroprotective properties of A. pyrethrum can be attributed to its antioxidant and anti-inflammatory activities [63]. The phytochemical screening of A. pyrethrum revealed the presence of saponins, terpenoids, flavonoids, tannins, and alkaloids, and those secondary metabolites enhance GABA transmission [21,64,65]. Moreover, the biochemical screening of the roots of A. pyrethrum, showed they contain principally pellitorine as a main active constituent, which is a N-isobutyl amide alkaloid [66]. Alkaloids, including pellitorine, exhibit antioxidant properties and can contribute to the reduction of oxidative stress through multiple mechanisms. These alkaloids can directly scavenge ROS, neutralizing them and preventing oxidative damage to cells. In addition, alkaloids have been shown to stimulate the activity or expression of endogenous antioxidant enzymes, which play a vital role in protecting cells from oxidative stress. Moreover, alkaloids can modulate signaling pathways, including those involving dopamine and GABA, which are neurotransmitters involved in various physiological functions. Dopamine and GABA have been implicated in antioxidant defenses and neuroprotection, and their interaction with alkaloids may further enhance the cellular antioxidant response. Furthermore, alkaloids may influence the balance of GABAergic neurotransmission, which can impact oxidative stress levels. By reducing oxidative stress, alkaloids, in combination with dopamine and GABA, have the potential to support cellular well-being and protect against oxidative damage [67,68].

In summary, the combination of A. pyrethrum’s anxiolytic and antidepressant properties, along with its antioxidant and anti-inflammatory activities, holds promise for the management of various aspects of Clonazepam dependence.
CONCLUSION

In conclusion, our study provides compelling evidence that exposure to Clonazepam leads to depression-like behavior and biochemical alterations accompanied by increased oxidative stress levels. However, the administration of *A. pyrethrum* aqueous extract effectively mitigated the dependence-like behavior induced by Clonazepam. Importantly, the ability of AEAP to modulate oxidative stress is considered one of the contributing factors to its protective effects on Clonazepam-exposed rat stress in the brain. These findings highlight the potential of *A. pyrethrum* as a promising therapeutic approach for managing the adverse effects of Clonazepam dependence, by attenuating oxidative stress-induced damage; it represents a novel and natural alternative for alleviating the unfavorable consequences associated with prolonged Clonazepam use. Further investigation is required to gain a deeper understanding of the molecular mechanisms responsible for the observed effects and to fully explore the therapeutic potential of *A. pyrethrum* in addressing SUD.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

All procedures involving animals were carried out in compliance with the guidelines outlined by the European Council Directive for Care and Use of Laboratory Animals (EU2010/63). Before the commencement, the Institutional Local Review Board approved the study protocol for animal experimentation, with the protocol code CA965/07/22 and an approval date of October 2022.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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