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Evaluation of Efficacy and Safety of Aqueous Root Extract of *Cannabis sativa* for Alcohol Use Disorder (AUD) in Wistar Albino Rats

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Abstract

Background: Alcohol Use Disorder (AUD) significantly impacts physical and neurological health. Existing pharmacotherapies face limitations due to adverse effects, high relapse rates, and poor patient adherence, driving interest in plant-based therapies with better safety profiles. Despite *Cannabis sativa's* historical medical use, limited research exists on its root extract, which may offer neuroprotective and hepatoprotective benefits. **Aim:** To evaluate the efficacy, safety and neuroprotective effect of aqueous root extract of *C. sativa* in a rat model of AUD. **Methods:** 30 Wistar rats were divided into five groups (n=6): normal control, AUD control, *C. sativa* extract (low dose-25mg/kg and high dose-50mg/kg), and standard (acamprosate). Over 21 days, a 24-hour two-bottle choice paradigm was employed. From day 1-7, both bottles were filled with drinking water. From day 8-21, one bottle was filled with Ethanol and the other with drinking water, along with the respective treatments. Ethanol intake and preference were measured daily in all the groups. Haematological, biochemical tests and behavioural assessments were performed on day 21, following which the rats were sacrificed, and histopathological analysis of vital organs was performed. Phytochemical analysis of the extract were done using chemical tests and GC-MS. *In vitro* antioxidant and anti-inflammatory effects were assessed using the DPPH reduction assay and Egg albumin denaturation assay. **Results:** *C. sativa* significantly decreased ethanol intake and preference, and alleviated the neurological symptoms of AUD, with a good safety profile. The high-dose extract exhibited comparable efficacy with acamprosate. Histopathological analyses revealed reduced organ damage, with near-normal architecture in the high-dose group. The extract demonstrated promising antioxidant and anti-inflammatory effects. **Conclusion:** The findings of this research show that *C. sativa* is a potential therapeutic option in AUD, demonstrating notable hepatoprotective and neuroprotective effects in the rat model of AUD, which can be further explored in clinical studies.

Major Findings: High-dose *C. sativa* reduced ethanol intake/preference and improved behavioural outcomes comparable to acamprosate. It also mitigated AUD-related organ damage and showed *in vitro* antioxidant and anti-inflammatory activity.

Keywords: Alcoholism, Alcohol Use Disorder, *Cannabis*, Hepatoprotective, Neuroprotective

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1. Introduction

Alcohol Use Disorder (AUD) is a prevalent condition with severe global public health and socio-economic implications[1]. According to the World Health Organisation (WHO), excessive alcohol consumption leads to about 3 million deaths annually, representing nearly 5% of total deaths worldwide[2]. AUD is characterised by persistent alcohol-seeking actions, diminished ability to control intake, and the development of adverse emotional states upon cessation[3]. Long-term alcohol use inflicts damage on vital organs, such as the liver, brain, and heart, while also contributing to mental health challenges, social disruption, and economic burdens for families and healthcare systems. Despite the presence of established medications like naltrexone, disulfiram, and acamprosate, treating AUD remains challenging[4]. Barriers such as poor patient compliance, undesirable side effects, high relapse rates, and variable patient responses to medication highlight the need for alternative therapies. There is growing interest in exploring plant-derived medicines due to their broad range of effects, lower toxicity, and cultural acceptance.

Historically, medicinal plants have been used to manage neurological and psychiatric disorders, including substance dependence. Many plant-based compounds are noted for their neuroprotective, antioxidant, anxiolytic, and anti-craving benefits[5]. Using plant extracts presents a promising direction for developing new treatments for AUD[6]. Roots of certain medicinal plants have long been employed in traditional medicine for addressing behavioural issues, neurological conditions, and detoxification, but scientific validation of these practices is limited. *C. sativa*, commonly known as hemp, stands out as a medicinal plant with a wide spectrum of pharmacological activities. Its constituents—cannabinoids (such as cannabidiol and tetrahydrocannabinol), terpenoids, flavonoids, and alkaloids—produce various effects on the central nervous system, including anti-anxiety, antidepressant, anti-inflammatory, and neuroprotective actions[7-9]. Research has mainly focused on the aerial parts of *C. sativa*, particularly the leaves and flowers, due to their higher tetrahydrocannabinol levels, while the roots have received less scientific attention. Traditionally, *C. sativa* roots have been used for relieving pain, inflammation, fever, and digestive issues[10]. Early phytochemical studies have revealed the presence of compounds like friedelin, pentacyclic triterpenes, and other secondary metabolites in the roots. These substances are believed to possess analgesic, sedative, and hepatoprotective properties, which may be beneficial in managing AUD and its complications[11].

The endocannabinoid system (ECS) is recognised for its significant role in regulating reward, motivation, and addictive behaviors[12]. The alcohol-seeking behaviour is demonstrated to be influenced by the phyto cannabinoids' modulation of the endocannabinoid system, and it was shown to minimise relapse tendencies in preclinical research[13]. Investigating the aqueous extract is especially pertinent for traditional medicinal systems that frequently utilise water-based decoctions or infusions. With this background, this study was planned to examine the effectiveness and safety of *C. sativa* aqueous root extract utilising a validated Wistar albino rat model of alcohol use disorder.

The present study was undertaken to evaluate the efficacy of aqueous root extract of *C. sativa* in an animal model of alcohol use disorder, with the primary objective of determining its effect on ethanol intake and ethanol preference in rats.

2. Materials and Methods

2.1 Preparation of Plant Extract

The high-quality *C. sativa* roots were collected from the Solan district of Himachal Pradesh, India, and identified by the Botanical Survey of India, High Altitude Western Himalayan Regional Centre, Nauni, Solan, Himachal Pradesh, India, with accession no. 0697. 10 grams of *C. sativa* roots were ground into fine powder using an electrical grinder, added to 100 ml of distilled water and placed in a shaking incubator for 48 hours at 37°C. Later, the supernatant was filtered out using Whatman filter paper. The obtained solution was further kept in a shaking incubator at 37°C to obtain a thick crude extract and refrigerated at 4°C. The same procedure was followed several times to obtain the required amount of the crude extract for further utilisation.

2.2 *In Vivo* Animal Study

The *in vivo* study was conducted after attaining proper ethical approval from the Institutional Animal Ethics Committee (Ref no: IAEC 1 / Proposal: 154/A.Lr:116/

Dt: 20:02:2024). The research utilised thirty healthy adult male Wistar albino rats, each weighing between 150 and 250 grams. The animals were acquired from a verified vendor and maintained under standard laboratory conditions, comprising a 12-hour light/dark cycle, temperature (22 ± 2°C), and relative humidity (55 ± 5%). The experiment started after an acclimatisation period of one week (7 days). Throughout the experimental period, the rats had unrestricted access to a standard pellet diet and clean drinking water.

2.3 Induction of Alcohol Use Disorder (AUD)

The 24-hour Two Bottle Choice (TBC) method was used in this study as a rodent drinking model of alcoholism, as described by Huynh, Nhat, *et al.*[14], where the rats were housed individually in a cage fitted with two bottles of drinking water. After a quarantine period of one week, the rats were given drinking water and 10% Ethanol (v/ v) (500 mL solution prepared by adding 50 mL of 95% ethanol and 450 mL of water) for 21 days. From day 1-7, both bottles were filled with drinking water. From day 8-21, one bottle was filled with drinking water and the other with 10% Ethanol. To overcome the conditioned place preference in rats, which might influence drinking activity, the position of the two bottles was alternated every other day.

2.4 Study Design and Grouping

The animals were randomly assigned to five experimental groups 1-5, with 6 rats per group. Group 1 served as the Normal control and was not administered ethanol or treatment. In groups 2-5, AUD was induced using the TBC method, as explained above, from day 8 to 21.

The grouping of animals and group-wise interventions given from days 8 to 21 is shown in Table 1.

2.5 Study Assessments

Ethanol intake (mL), Ethanol intake (g/kg) and Ethanol preference (%) were recorded daily from day 8 till day 2, in groups 2 to 5, using the following formulae[15-17]:

$$Ethanol\ Intake(mL) = [Volume\ of\ ethanol_{current\ day} - Volume\ of\ ethanol_{previousday}] - \text{Mean ethanol loss from control cages}$$

$$Ethanol\ Intake(g/kg) = \frac{Ethanol\ Intake(mL) \times 0.7893\,g/mL}{Body\ Weight(kg)}$$

$$Ethanol\ Preference(\%) = \frac{Ethanol\ Intake(mL)}{Water\ Intake(mL)} \times 100$$

CBC, RFT, LFT and Behavioral assessments (EPM, TST and OFT) were done on day 21 in all the groups, after which the rats were sacrificed, and vital organs (Brain, Kidney, Liver and Heart) were removed, and histopathological analysis of the same was done.

In addition, body weight measurement was done at baseline and then once a week. Throughout the study, signs of toxicity and mortality in rats were observed daily.

2.6 Behavioural Assessment

Behavioural assessments were conducted towards the end of the study.

2.6.1 *Elevated Plus Maze (EPM) Test - Assessment of Anxiety-like Behaviour*

The EPM apparatus consists of four arms (2 open and 2 closed) with a central platform, placed at a height of 50 cm above the ground level. Rats were placed in the central platform, and the number of entries made into the open arms and the time spent in the open arms were noted to evaluate the anxiety levels. The readings were recorded over 5 minutes for each rat[18].

Table 1. Grouping of animals and interventions

Groups		Intervention
Group 1	Normal control	AUD was not induced, and no treatment was given
Group 2	AUD control	AUD + vehicle (distilled water given through oral gavage)
Group 3	Standard control	AUD + Standard drug (Acamprosate 200 mg/kg/ day in distilled water, given through oral gavage)
Group 4	*C. sativa* low dose	AUD + *C. sativa* root extract (25 mg/kg in distilled water, given through oral gavage)
Group 5	*C. sativa* high dose	AUD + *C. sativa* root extract (50 mg/kg in distilled water, given through oral gavage)

2.6.2 Tail Suspension Test (TST) - Assessment of Anti-depressant Activity

TST is a commonly used screening assessment for antidepressant activity based on the concept of depressive behaviour. The rats are suspended by their tails for 6 minutes, and the duration of immobility, i.e. the absence of escape behavior is assessed, which serves as the primary measure of depressive-like behaviour. Increased immobility indicates greater behavioural despair, while reduced immobility suggests antidepressant-like effects. This test is particularly relevant for alcohol use disorder as major depressive episodes are highly associated with AUD, occurring in up to 40% of patients[19,20].

2.6.3 Open Field Test (OFT) - Assessment of Locomotor Activity and Anxiety-related Exploration

Locomotor activity and anxiety-related behaviours were evaluated using OFT in a controlled setup. The apparatus consists of a large square space (100×100 cm) divided into peripheral and central zones. The primary anxiety measure parameter was the time spent in the centre zone, as rodents naturally avoid open, exposed areas. Increased central zone exploration indicates reduced anxiety and normal exploratory behaviour, while decreased central zone time suggests elevated anxiety or compromised exploratory interest. General Locomotor activity is assessed along with anxiety assessment[21,22].

2.7 Physiological and Biochemical Parameters

Physiological parameters, namely, body weight and mortality, were recorded daily during the entire duration of the study. Haematological and biochemical evaluations were conducted at baseline (day 1) and on the final day (day 21). Blood samples (0.5 mL) were obtained using retro-orbital puncture under mild anaesthesia. The evaluated parameters encompassed complete blood count (CBC), renal function tests (urea and creatinine), and liver function tests (AST, ALT, and ALP) to ascertain systemic toxicity or organ impairment.

2.8 Histopathological Analysis

At the end of the study (day 21), all rats were humanely euthanised via high-dose halothane anaesthesia. Essential organs, namely, the brain, liver, kidneys and heart were dissected out and stored in 10% formalin for histopathological examination. Tissue slices were processed, stained, and analysed microscopically for cellular changes suggestive of alcohol-induced harm or treatment-related protective effects.

2.9 Phytochemical Screening

Screening for the presence of various phytochemicals was done using relevant chemical tests[23-33].

2.10 Assessment of Antioxidant Activity (DPPH Reduction Assay)

"DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay was used in evaluating the antioxidant activity. A mixture of 1 mL of 0.4% DPPH solution and 1 mL of the test sample (extract) was taken in a test tube. This reaction mixture was incubated for 30 mins in a dark place. After incubation, the absorbance was measured at 517 nm. The radical scavenging activity was measured as a decrease in the absorbance of DPPH. The DPPH radical scavenging capacity was estimated from the difference in absorbance for the drug and the control blank. In this assay, the control typically shows higher absorbance at 517 nm. The data were presented as a percentage of DPPH scavenging. IC_{50} (concentration of extracts resulting in 50% reduction of the DPPH radical) values were calculated using linear regression analysis with GraphPad Prism software.

"DPPH Scavenging activity (%) = (Abs of control – Abs of sample)/Abs of control x 100"

The experiment was performed with a standard antioxidant compound, ascorbic acid and "AEAC (Ascorbic Acid Equivalent Antioxidant Capacity) was calculated for the test sample with the formula, (IC_{50} of Ascorbic acid / IC_{50} of test sample) x 100.

2.11 Assessment of Anti-inflammatory Activity (Egg Albumin Denaturation Assay)

The egg albumin denaturation method was used to evaluate the anti-inflammatory activity. The reaction mixture (5 mL) consisted of 0.2 mL of egg albumin (from fresh hen's egg), 2.8 mL of phosphate-buffered saline (PBS, pH 6.4) and 2 mL of varying concentrations of test sample. A similar volume of double-distilled water served as the control. The prepared solutions were

incubated at 37±2°C for 15 minutes and then heated at 70°C for 5 minutes. Upon cooling, their absorbance was measured at 660 nm. Diclofenac sodium was used as the reference drug. The percentage inhibition of protein denaturation was calculated by using the following formula:

"% inhibition = $100 \times [(V_t / V_C) - 1]$"

Where,

"V_t = absorbance of the test sample"

"V_c = absorbance of control".

2.12 Statistical Analysis

The values were summarised as mean ± standard deviation. GraphPad Prism 7.0 software (La Jolla, CA, USA) was used for Statistical analyses. "One-way ANOVA followed by Tukey's multiple comparison test was employed for Group comparisons". Statistical significance was deemed to be present when $p < 0.05$ at all instances.

3 Results

3.1 Ethanol Intake in mL/day

Ethanol intake in mL/day was calculated in all the rats of groups 2-4 and compared across three time points, day 8, day 15 and day 21. The results are presented in Figure 1-I. AUD control rats had the highest ethanol intake, in terms of mL/day, throughout the study period from day 8 to 21. Treatment with Acamprosate (200 mg/kg/day) resulted in a significant reduction in the Ethanol intake compared to the AUD control at all time points (day 8, day 15 and day 21). The mean Ethanol intake in ml/day in Acamprosate rats was 18.0 ± 1.4 mL on day 8, 11.7 ± 1.0 mL on day 15, which further decreased to 6.5 ± 0.5 mL on day 21.

The *C. sativa* root extract-treated groups exhibited dose-dependent reductions in ethanol intake. The low-dose group (25 mg/kg/day) showed a progressive decrease from 17.8 ± 1.5 mL on day 8 to 11.2 ± 0.9 mL on day 21. Significant reductions compared to AUD controls were observed at day 15 (mean difference ≈ 5.3 mL; $P < 0.0001$) and day 21 (mean difference ≈ 10.0 mL; $P < 0.0001$). The high-dose *C. sativa* root extract (50 mg/kg/day) exhibited a stronger response, reducing ethanol intake from 18.2 ± 1.6 mL on day 8 to 8.5 ± 0.6 mL on day 21. Significant reductions relative to AUD controls were similarly observed at day 15 (mean difference ≈ 7.2 mL; $P < 0.0001$) and day 21 (mean difference ≈ 12.7 mL; $P < 0.0001$). Furthermore, the high-dose group showed statistically significant ethanol intake reductions compared to the low-dose group on day 21 (mean difference = 3.8 mL; $P < 0.0001$), affirming a dose-dependent effect. Intragroup comparison from day 8 to day 21 within the *C. sativa* groups revealed significant reductions for the high-dose group ($P < 0.0001$), whereas changes within the low-dose group were nonsignificant ($P = 0.66$). The reduction in ethanol intake on day 21 showed no difference between Acamprosate and high-dose extract.

3.2 Ethanol Intake in g/kg

The assessment of Ethanol intake in grams per kg body weight of the rats, as depicted in Figure 1-II, revealed that Acamprosate significantly reduced Ethanol intake at all time points relative to AUD controls, with values declining from 5.12 ± 0.45 g/kg (day 8) to 3.31 ± 0.28 g/kg (day 15) and further to 2.06 ± 0.16 g/kg (day 21) and the reduction was statistically significant from day 8 to day 21 ($P<0.0001$).

Cannabis sativa root extract showed dose-dependent reduction in ethanol consumption. 25 mg/kg extract-treated rats exhibited a significant reduction in the mean intake of ethanol of 4.98 ± 0.44 g/kg (day 8), 4.07 ± 0.37 g/kg (day 15), and 2.76 ± 0.24 g/kg (day 21). The 50 mg/kg group displayed further reductions, with 5.09 ± 0.45 g/kg (day 8), 3.46 ± 0.26 g/kg (day 15), and 2.20 ± 0.16 g/kg (day 21). On day 21, the ethanol intake in g/kg body weight was similar for both 25 mg/kg and 50 mg/kg extract.

Between-group comparisons at each time point demonstrated that all treatment groups had significantly lower ethanol intake than the AUD group at day 15 and day 21 ($P < 0.0001$ for both doses of *C. sativa* extract and Acamprosate). Notably, the reductions achieved with *C. sativa* (50 mg/kg) were comparable to those with Acamprosate at day 21 ($P = 0.99$, not significant), further substantiating the efficacy of the high dose.

3.3 Ethanol Preference (%)

The data is represented in Figure 1-III. The AUD control group demonstrated a high and increasing ethanol preference, with means of 52.9 ± 4.8% on day 8, 55.6 ± 3.3% on day 15, and 60.1 ± 2.4% on day 21, indicating strong and sustained alcohol preference.

Acamprosate-treated rats demonstrated a significant reduction in ethanol preference from day 8 to day 21. In addition, the ethanol preference (%) was significantly less compared to AUD control at all time points compared to AUD controls.

Cannabis sativa root extract showed a dose-dependent decrease in ethanol preference. Ethanol preference (%) in the low-dose group (25 mg/kg) decreased from 50.9 ± 4.5% on day 8 to 41.1 ± 4.1% on day 15 and 29.3 ± 2.8% on day 21. The high-dose group (50 mg/kg) exhibited a more marked decline from 52.0 ± 4.3% at day 8 to 34.3 ± 3.2% by day 15 and 17.2 ± 2.1% at day 21. Within-group analysis showed a significant reduction across the time points for both doses.

Between-group analysis revealed that both doses of *C. sativa* root extract and Acamprosate significantly lowered ethanol preference compared to AUD controls at days 15 and 21 ($P < 0.0001$). Notably, the high-dose *C. sativa* group's ethanol preference at day 21 was comparable to the Acamprosate group (mean difference = 1.6%, $P = 0.99$), indicating similar efficacy in reducing alcohol preference.

3.4 Behavioural Assessments

Three behavioural paradigms for assessing anxiety-like behaviour (Elevated Plus Maze), depressive-like behaviour (Tail Suspension Test), and exploratory behaviour with anxiety (Open Field Test) were done at the end of the study (day 21) in all the groups.

3.4.1 Elevated plus maze test (Figure 2-I)

Rats in the control group spent a mean of 40.22 ± 3.02 seconds in the open arms, representing baseline anxiety levels consistent with normal, exploratory behaviour in rats. The mean time spent in the open arms in the AUD control group was 11.91 ± 4.90 seconds, a statistically significant 70% decrease compared to the normal control ($p < 0.001$), establishing a significant anxiety behaviour in AUD. Acamprosate Group had a notably increased open-arm time to (33.63 ± 6.45 seconds). This improvement was highly significant compared to the AUD control ($p < 0.001$), amounting to approximately 76.7% recovery toward the normal group.

Rats in the *C. sativa* high dose (50 mg/kg) spent 28.12 ± 4.63 seconds in open arms, thereby signifying a highly significant improvement over the AUD group ($p < 0.001$) and had attained comparable therapeutic efficacy to acamprosate ($p = 0.591$).

The *C. sativa* low-dose (25 mg/kg) group demonstrated mild anxiolytic effects (25.52 ± 4.96 seconds spent in open arms). The improvements were highly significant versus AUD control ($p = 0.002$), with 75.9% of the efficacy of acamprosate. Though there was a dose-response trend favouring the 50 mg/kg dose over 25 mg/kg ($p = 0.182$), the difference was not statistically significant.

3.4.2 Tail Suspension Test(Figure 2-II)

The normal control group elicited a mean immobility time of 62.41 ± 10.91 seconds during the 6-minute TST session, indicating a normal behavioural despair response. The AUD group had an increased mean immobility time of 115.75 ± 9.07 seconds, which implies a highly significant 85.5% increase in depressive-like behaviour versus the normal control group ($p < 0.001$). Acamprosate-treated rats showed a significant reduction in the immobility with a duration of 66.72 ± 2.06 seconds, which was 42.3% less compared to the AUD control group ($p < 0.001$) and similar to the normal control group ($p = 0.820$), indicating a near-normal behaviour and good antidepressant-like activity. *C. sativa* high dose (50 mg/kg) group led to moderate antidepressant-like effects; immobility

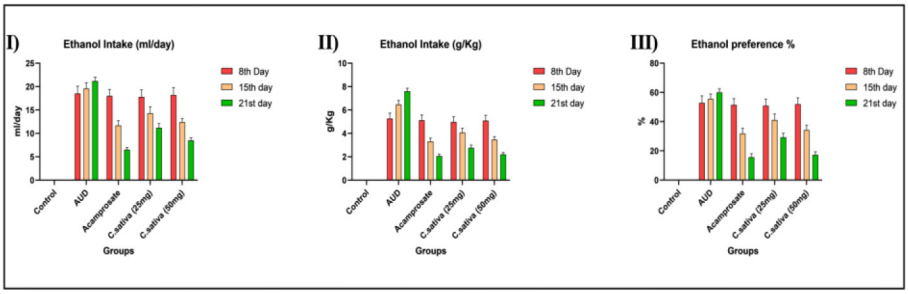


Figure 1. Ethanol intake, values are presented as mean (n=6).

was reduced to 87.43 ± 10.81 seconds, which was a significant 24.5% decrease compared to the AUD control group (p = 0.001), but remained high compared to acamprosate-treated groups (p = 0.001). A similar effect on immobility time in TST was exhibited by *C. sativa* low-dose (25 mg/kg) treated rats.

3.4.3 Open Field Test (Figure 2-III)

The normal control rats exhibited typical exploratory behaviour and low anxiety, spending 45.94 ± 5.17 seconds in the central zone (≈15% of the total session). In contrast, markedly impaired exploratory activity was demonstrated by the AUD control group, with centre time reduced to 13.12 ± 7.55 seconds. This represents a 71.4% decrease compared to normal controls (p < 0.001). Treatment with the standard control-acamprosate regained exploratory activity, with the rats spending 42.77 ± 2.92 seconds in the centre, pertaining to no significant difference from the normal control group (p = 0.96). This indicates a near-complete normalisation of anxiety-like behaviour. *C. sativa* extract (50 mg/kg) produced a significant improvement, with 34.57 ± 3.52 seconds in the centre (p < 0.001). The low dose (25 mg/kg) group exhibited moderate efficacy, with 25.56 ± 6.16 seconds spent in the centre (p = 0.006), yet it was significantly lower compared to control and acamprosate (p < 0.001). Superior behavioural recovery with the higher extract dose (p < 0.001) was confirmed by the direct dose–response analysis.

3.5 Haematological and Biochemical Observations

3.5.1 Complete Blood Count

Across all intervention groups, including the standard and test dose extract groups, the blood indices remained stable. There were no significant differences compared to acamprosate or from controls. A slight elevation in WBC (7.54 ± 0.94 × 10^3/µL) compared to acamprosate (7.41 ± 0.69 × 10^3/µL) was noted in the AUD group alone, but this elevation was not statistically significant (p > 0.05). These readings suggest that there is no clinically relevant inflammatory response among treated groups (Figure 3- I to V).

3.5.2 Renal Function Tests

The AUD group developed pronounced renal stress, reflected in increased BUN (33.55 ± 2.80 mg/dL) and creatinine (2.91 ± 0.39 mg/dL). Acamprosate treatment significantly normalised these markers (BUN: 20.35 ± 0.75 mg/dL; creatinine: 1.55 ± 0.17 mg/dL), resulting in values closely resembling normal controls (p > 0.05 for

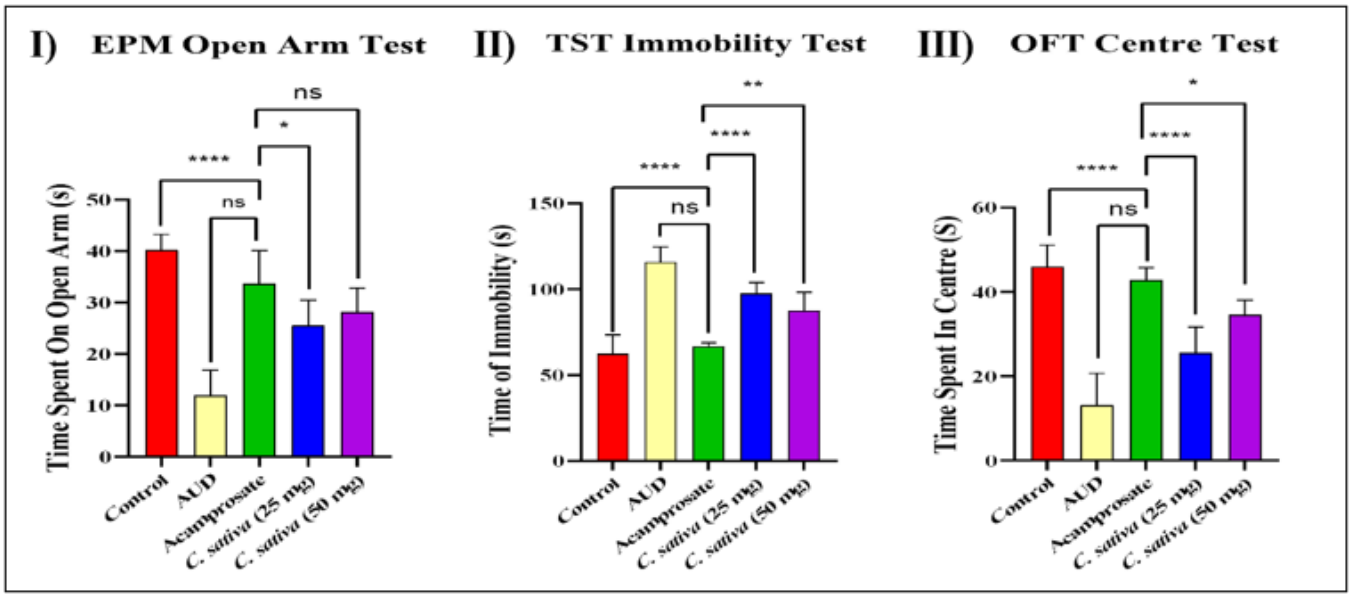


Figure 2. Behavioural tests**.** Values are presented as mean (n=6), ****p<0.0001, ***p<0.001, **p<0.01 and ns is for non-significant.

BUN, $p < 0.05$ for creatinine), denoting near-complete nephroprotection. The high-dose *cannabis* group (50 mg/kg) also provided significant renal protection (BUN: 21.53 ± 1.10 mg/dL, creatinine: 1.68 ± 0.15 mg/dL), but both parameters remained marginally higher than those observed in the acamprosate group ($p > 0.05$), suggesting a comparable effect on BUN but slightly less so for creatinine. The low-dose group had a lesser reno-protective effect and did not match either the standard or high-dose extract group (Figure 3- IX, X).

3.5.3 Liver Function Tests

The AUD group showed marked hepatocellular injury, evidenced by significant elevations in ALT (57.84 ± 2.79 U/L), AST (121.14 ± 9.72 U/L), and ALP (360.54 ± 17.33 U/L). In contrast, the acamprosate group demonstrated substantial hepatoprotection with ALT (32.18 ± 4.52 U/L), AST (74.24 ± 5.63 U/L), and ALP (239.34 ± 26.22 U/L), which were significantly lower than the AUD group ($p < 0.0001$ for ALT, AST and ALP). The values in the acamprosate group approached normal controls but remained modestly elevated (all $p < 0.05$ vs. control), indicating effective but incomplete recovery. *C. sativa* root extract produced dose-dependent improvements. At 50 mg/kg, ALT (39.80 ± 3.94 U/L), AST (90.76 ± 7.99 U/L), and ALP (254.85 ± 15.04 U/L) were all significantly decreased compared to the AUD group ($p < 0.001$), yet remained higher than the acamprosate group for ALT and AST ($p < 0.05$ for both). ALP values in the 50 mg group were comparable to those of the acamprosate group ($p > 0.05$), suggesting a near-equivalent effect for this parameter. The low-dose extract group (25 mg/kg) showed smaller reductions and remained statistically inferior to both acamprosate and high-dose *cannabis* (Figure 3- VI to VIII).

3.6 Histopathological Analysis of Vital Organs

Histopathological examination of vital organs, performed on day 21, demonstrated significant tissue alterations among the experimental groups. The "Haematoxylin and Eosin (H and E)" stained, representative micrographs of tissue sections from each experimental group are shown at 10× magnification (Figure 4). The control group showed normal morphology in the kidney, liver, heart, and brain, with no detectable pathological changes. Conversely, the AUD control group exhibited substantial tissue damage in the vital organs. The kidneys revealed pronounced vascular congestion; the heart presented with congested blood vessels and intramuscular congestion; the liver displayed hypertrophied hepatocytes, sinusoidal congestion, and mild periportal lymphocytic infiltration; and the brain manifested extensive oedema, neuronal degeneration, microcystic alterations, and disorganisation, characterised by clustering of astrocytes, astrogliosis, and disarrayed cerebellar nuclei.

In Acamprosate-treated rats, renal architecture was preserved, although cardiac tissue displayed congestion and dilated capillaries, hepatic tissue had feathery degeneration accompanied by nuclear enlargement and binucleation, while cerebral areas revealed moderate neuronal degeneration, mild oedema, and pronounced astrogliosis.

Cannabis sativa root extract exhibited partial amelioration, characterised by modest renal congestion, a limited number of clogged cardiac arteries, feathery degeneration in the liver accompanied by little inflammation, and less neuronal damage and gliosis in the brain. *C. sativa* root extract exhibited significant histological recovery, with kidney and heart tissues appearing unremarkable, liver sections displaying mild sinusoidal dilation, hepatocyte binucleation, and degeneration, while brain sections revealed only mild neuronal degeneration, moderate astrogliosis, and elevated capillary density. The aqueous root extract of *C. sativa* exhibited dose-dependent organ protective effects, with the high-dose group significantly preserving hepatic and neuronal architecture, indicating hepatoprotective and neuroprotective efficacy against alcohol-induced toxicity in Wistar albino rats.

3.7 Phytochemical Screening

The presence of "phenols, flavonoids, glycosides, terpenoids, alkaloids, carbohydrates and proteins" was revealed over the phytochemical analysis of *cannabis* root extract.

3.7.1 Gas Chromatography- Mass Spectrometry (GC-MS)

A total of 9 peaks corresponding to bioactive compounds were identified on GC–MS chromatogram of root extract of *C. sativa* (Figure 5 and Table 2). These compounds were identified by comparing parameters

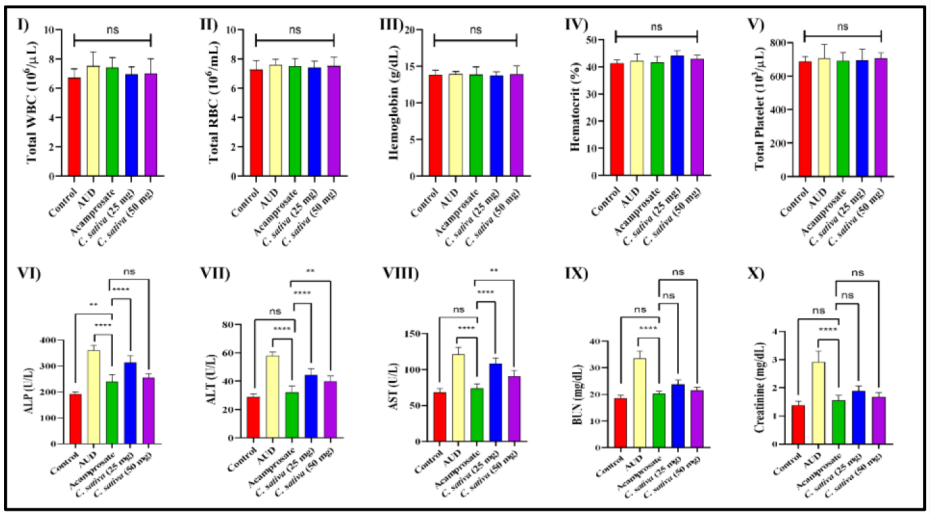


Figure 3. Haematological and Biochemical parameters (I) WBC Total count, (II) RBC Total count, (III) haemoglobin level, (IV) hematocrit level, (V) total platelet count, (VI) ALP level, (VII) ALT level, (VIII) AST level, (IX) BUN level and (X) creatinine level. Values are presented as mean (n=6), "****p<0.0001, ***p<0.001, **p<0.01 and stands for non-significant.

such as peak retention time, peak area (%), height (%), and mass spectral fragmentation patterns with those of known compounds cataloged in the National Institute of Standards and Technology (NIST) library[34-36].

3.8 Antioxidant activity

DPPH radical scavenging assay was used to assess the antioxidant potential of *Cannabis* root extract (Figure 6: A–C). As stated in Figure 6A, the root extract elicited a concentration-based increase of DPPH radical scavenging activity. At "100, 200, 400, 600, 800, and 1000 µg/mL concentrations" the scavenging activities were 55.25%, 58.91%, 62.71%, 66.34%, 76.23%, and 79.12%, respectively. In comparison, the standard antioxidant ascorbic acid ("1.5–2.5 µg/mL") showed 57.29%–61.63% scavenging activity. The IC_{50} value (required concentration to inhibit 50% of DPPH radicals) was calculated for both test and standard compounds. The *cannabis* root extract demonstrated an "IC_{50} of 535.4 µg/mL" (Figure 6B), whereas the standard ascorbic acid exhibited a much lower IC_{50} of 0.1454 µg/mL (Figure 6 C), indicating its higher antioxidant potency.

3.9 Anti-inflammatory Activity

Cannabis sativa root extract's anti-inflammatory activity was evaluated and compared with the standard anti-inflammatory drug diclofenac based on percentage inhibition effects (Figure 7). At 100 µg/mL, the extract showed 18% denaturation inhibition, while diclofenac exhibited 30% inhibition, with no significant difference. At higher concentrations, the extract demonstrated a dose-dependent increase in inhibitory activity. At 200 and 300 µg/mL, the extract showed 43% and 54% inhibition, respectively, which were significantly lower than diclofenac (73% and 77% inhibition) with statistical differences ($p < 0.01$ and $p < 0.05$, respectively). At 400 µg/mL, the inhibition values were 77% (extract) and 81% (diclofenac), showing no significant difference. Similarly, at 500 µg/mL, the extract exhibited 90% inhibition, which was comparable to diclofenac (97% inhibition), again showing no significant difference. These indicate that *C. Sativa* root extract exerts considerable concentration-dependent anti-inflammatory effects. Although it is less potent than diclofenac at lower concentrations, its activity approaches that of the standard drug at higher concentrations, suggesting promising anti-inflammatory potential.

4. Discussion

Alcohol Use Disorder (AUD) is notoriously difficult to manage because currently approved

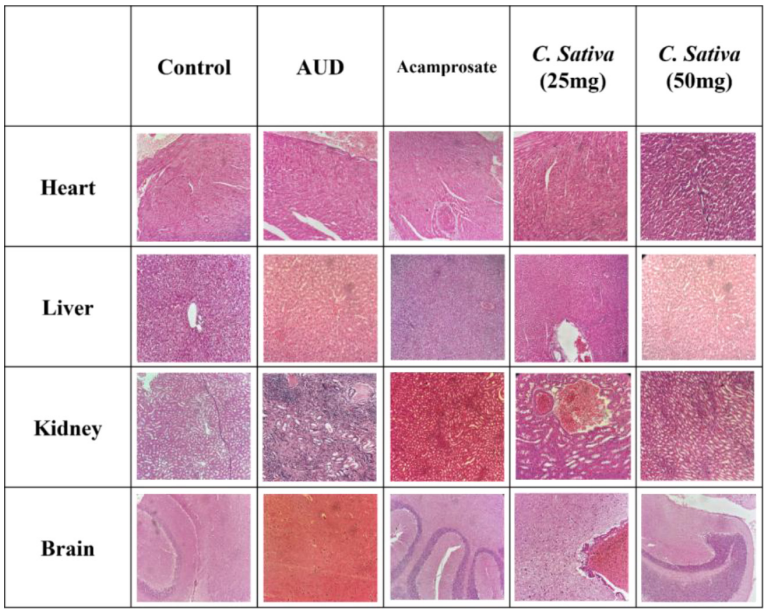


Figure 4. Histopathology of vital organs.

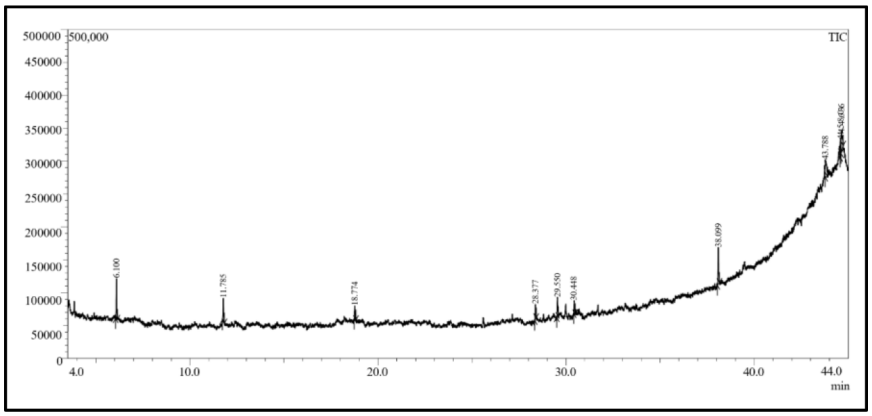


Figure 5. GC-MS analysis of the extract.

Table 2. Phytochemical identification using GC-MS

Sr. No.	Compound CID	Compound Name	Molecular Formula	Molecular Weight (g/mol)	Peak Area (%)
1	7641	"Hexanedioic acid, bis(2-ethylhexyl) ester"	"$C_{22}H_{42}O_{4}$"	"370.6"	15.65
2	3026	Dibutyl phthalate	$C_{16}H_{22}O_4$	278.34	15.41
3	12398	Heptadecane	$C_{17}H_{36}$	240.5	14.29
4	22212394	"2-hydroxy-7-methoxy-4-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl] oxy-1,4-benzoxazin-3-one"	"$C_{15}H_{19}NO_{10}$"	"373.31"	13.65
5	22211625	3alpha-(Trimethylsiloxy)cholest-5-ene	$C_{30}H_{54}OSi$	458.8	13.39
6	12389	Tetradecane	$C_{14}H_{30}$	198.39	8.85
7	123192	"4-dichloromethylene-1-methoxy-3,5-dimethyl-4h-phosphinine 1-oxide"	C_6Cl_8	355.7	7.53
8	644822	3-(13)propanenitrile	$C_{22}H_{35}N_3O$	357.5	5.78
9	11006	Hexadecane	$C_{16}H_{34}$	226.44	5.45

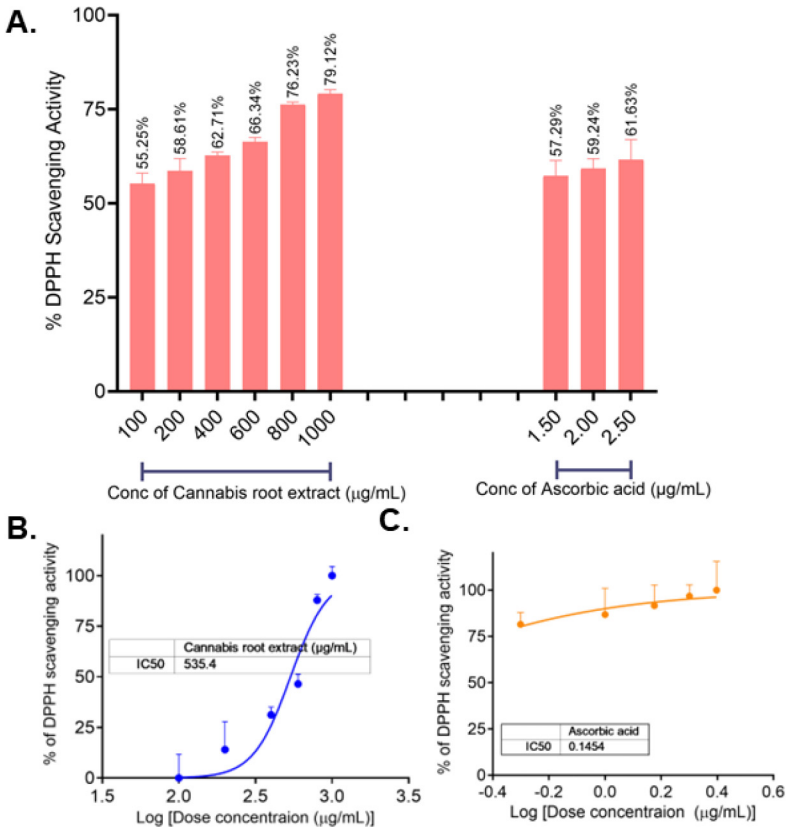


Figure 6. Antioxidant activity of the extract: *(A)*. DPPH radical scavenging, which is dose-dependent(%); *(B)*. IC_{50} value of *Cannabis* root extract using DPPH assay; *(C)*. IC_{50} of Ascorbic acid extract using DPPH assay.

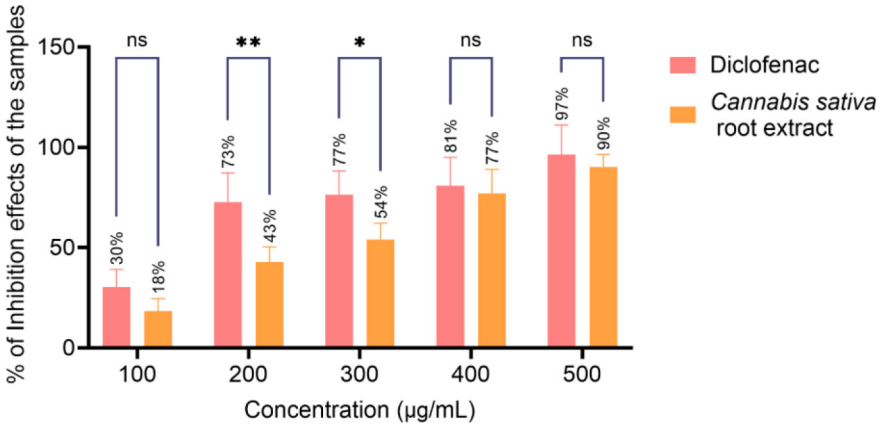


Figure 7. Anti-inflammatory activity of the extract.

pharmacotherapies, naltrexone, disulfiram and acamprosate, show limited long-term effect and are associated with high relapse rates. In this context, the potential of aqueous extract of *C. sativa* roots as an anti-craving, neuroprotective and hepatoprotective intervention is first evaluated in this research using a validated two-bottle-choice Wistar rat model of AUD. The key findings of this study are:

i. The dose-dependent decline in ethanol intake and preference.
ii. Marked improvement in anxiety- and depression-like behaviours.
iii. Partial normalisation of liver and brain histology.
iv. A good safety profile at both test dose-extract groups.
v. Demonstration of anti-inflammatory and antioxidant properties.

4.1 Efficacy in Reducing Ethanol Consumption

Administration of *C. sativa* root extract elicited a significant, dose-dependent decrease in ethanol intake and preference across the 21-day treatment period. The high dose (50 mg/kg), rats exhibited a ~50% reduction in daily alcohol intake compared to AUD controls, corresponding to ~81% of Acamprosate's efficacy, while the lower dose (25 mg/kg) achieved a ~35% reduction. When normalised to body weight (g/kg) and expressed as ethanol preference (%), both measures confirmed the robustness of the effect, with significant group differences emerging by day 15 and persisting through day 21. By the end of the study, the high-dose extract had converged with acamprosate in terms of g/kg intake and preference, indicating comparable clinical magnitude on key endpoints.

These results highlight that the aqueous root extract reliably attenuates alcohol consumption in a progressive, dose-dependent way. The 50 mg/kg dose approached a near-equivalent efficacy to the standard pharmacotherapy Acamprosate, while also introducing a botanical, multi-constituent alternative with translational promise.

GC–MS analysis identified DIMBOA-glucoside[37], trimethyl-siloxycholest-5-ene[38] and a chlorinated phosphinine derivative[39], each contributing complementary actions—antioxidant free-radical scavenging, neurosteroid-like modulation of GABA-A/NMDA receptors, and enzyme-linked anti-inflammatory effects, that collectively mitigate craving and withdrawal-linked excitotoxicity. Additionally, a nitrile-containing compound may modulate dopaminergic and serotonergic signaling[40], dampening relapse risk.

4.2 Neurobehavioural Outcomes

Broad anxiolytic and antidepressant-like effects of *C. sativa* root extract were consistently revealed by behavioural assays in a dose-dependent manner. In the EPM, ethanol-exposed rats exhibited pronounced open-arm avoidance, whereas high-dose extract increased open-arm exploration significantly, reaching ≈84% of Acamprosate's effect. In the TST, AUD rats demonstrated ≈85% higher immobility relative to controls; high dose extract reduced immobility by ≈53%, with low dose producing a moderate effect. In the OFT centre zone, time was suppressed by ≈71% in AUD rats. Acamprosate fully restored exploratory activity, while high-dose extract achieved ≈65% recovery, and low dose provided partial benefit.

Taken together, these findings demonstrate that *C. sativa* root extract ameliorates ethanol withdrawal-associated anxiety and depressive behaviours across complementary paradigms, with high-dose treatment approaching the efficacy of acamprosate. This effect could be due to the possible synergistic actions of its phytoconstituents on GABAergic, glutamatergic, and endocannabinoid pathways.

4.3 Organ-protective Effects and Safety Outcomes

Classical alcohol-induced lesions in the AUD group, such as sinusoidal congestion, periportal inflammation, hepatocyte damage, renal tubular degeneration, and neuronal oedema, were attenuated by the high dose extract. The changes were confirmed on histological examination. Preservation of hepatic, renal, cardiac, and neuronal architecture was on scale with behavioural recovery. In the biochemical aspect, the extract significantly reduced AUD-elevated ALT, AST, and ALP, comparable to Acamprosate levels for ALP and partially for ALT/AST. Similarly, BUN and creatinine elevations were normalised, with BUN indistinguishable from Acamprosate and creatinine showing near-complete recovery. Haematological values were maintained within normal limits, and no mortality or systemic toxicity was observed. Overall, the extract elicited an effective hepatoprotection, nephroprotection and favourable tolerability, likely mediated by its antioxidant and anti-inflammatory phytoconstituents.

4.4 Comparison with Previous *Cannabis*-AUD Studies

Only two preclinical reports have explored *Cannabis*-derived material in AUD models: Transdermal cannabidiol decreased stimuli-induced craving of alcohol seeking[41] and synthetic CB_2 agonists lowered operant ethanol self-administration[42]. Neither study addressed safety nor histopathology, nor did they examine root constituents. Conversely, our work demonstrates that a water-soluble root extract delivers anxiolytic, antidepressant, hepatoprotective

and neuroprotective effects with no detectable renal or cardiac toxicity, an outcome that broadens the therapeutic scope beyond single-target cannabinoid ligands.

4.5 Mechanistic Considerations

The endocannabinoid system (ECS) modulates reward salience, stress reactivity and glutamatergic homeostasis—processes central to alcohol dependence[43]. Phytocannabinoid-induced CB_1 partial agonism or CB_2 activation can reduce dopaminergic sensitisation to alcohol cues, while neurosteroid-like cholestane derivatives may dampen hyper-excitable GABA-A and NMDA receptor activity during withdrawal[44]. Concurrently, DIMBOA-glucoside's antioxidant action limits ROS-triggered neuroinflammation that exacerbates craving and relapse[45]. Together, these mechanisms plausibly explain the extract's multi-domain efficacy.

4.6 Limitations

The extract's phytochemical complexity does not show definitive contribution of effects to specific constituents; bioassay-guided fractionation may be needed. The study employed only male rats, leaving sex-specific responses unaddressed. The duration of the study is only 21 days, and longer post-treatment observation would clarify whether benefits persist and whether tolerance develops. Finally, CB_1/CB_2 antagonist studies could validate ECS involvement directly.

5. Conclusion

This study, which has been done on a rat model of alcohol use disorder, demonstrates that high-dose aqueous *C. sativa* root extract substantially reduces ethanol intake, while also alleviating anxiety- and depression-like behaviours. The extract achieved approximately 80% of the efficacy observed with the standard drug, Acamprosate, and delivered these beneficial outcomes without adverse effects on the kidney or heart. This confirms the favourable safety profile of the extract. Histopathological analysis further revealed significant hepatoprotective and neuroprotective effects, proved by the preservation of normal liver and brain tissue architecture in treated animals.

Importantly, the therapeutic effects of *C. sativa* root extract were dose-dependent, with the high-dose group yielding outcomes aligning with Acamprosate for reducing addictive behaviours and protecting vital organs. These multiple benefits, in conjunction with good tolerability, support the extract's promise as an alternative therapy for alcohol use disorder. Further pharmacological investigation and clinical research are essential to fully realise its therapeutic potential in treating AUD.

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Author Contribution

Conceptualisation - T.V., A.E. and A.R.; Methodology - T.V., Y.V. and A.E.; Investigation/experimental work - T.V. and A.E.; Data curation - T.V., A.E. and P.E.M.; Formal analysis - T.V., K.S. and V.K.D.; Writing—original draft preparation - T.V.; Writing—review and editing - A.E., V.K.D., S.K., P.E.M. and A.R.; Supervision - A.R. All authors have read and agreed to the published version of the manuscript.