PREPARATION AND PHARMACOLOGICAL EVALUATION OF A POLYHERBAL PREPARATION (POL-6) ON ALCOHOL DEPRIVATION EFFECTS FOLLOWING VOLUNTARY ALCOHOL CONSUMPTION IN RATS

Thesis submitted in fulfillment of the requirement for the Degree of

DOCTOR OF PHILOSOPHY

By

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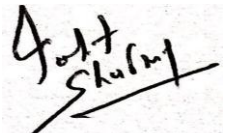
DECLARATION BY THE SCHOLAR

I hereby declare that the work reported in the Ph.D. thesis entitled "**Preparation and pharmacological evaluation of a polyherbal preparation (POL-6) on alcohol deprivation effects following voluntary alcohol consumption in rats"** submitted at **Jaypee University of Information Technology, Waknaghat**, **Solan (H.P.) India** is an authentic record of my work carried out under the supervision of **Dr. Gopal Singh Bisht** and **Dr. Girdhari Lal Gupta**. I have not submitted this work elsewhere for any other degree or diploma. I am fully responsible for the contents of my Ph.D. thesis.



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SUPERVISOR'S CERTIFICATE

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LIST OF ACRONYMS AND ABBREVIATIONS

%	Percentage
≤	Less than or equal to
≥	Greater than or equal to
◦C	Degree Celsius
α	Alpha
β	Beta
γ	Gamma
H	Hour
µg	micro gram
λ	Wavelength (Lambda)
θ	Theta
ml	milli liter
µl	Micro liter
yrs	years
g	gram
kg	Kilogram
◦C	Degree celcius
ALB	Albumin
ANOVA	Analysis of variance
BAC	Blood Alcohol Concentration
BDNF	Brain-Derived Neurotropic Factor
CB	Cannabinoid
CHOL	Cholesterol
CMC	Carboxymethyl cellulose
cDNA	Complementary DNA
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
DALYs	Disability-adjusted life years
DPPH	Diphenyl-2-picrylhydrazyl
DTs	Delirium tremens
DMA	Dorsal medial thalamus

EOS	Eosinophil
ESR	Erythrocyte sedimentation rate
EPM	Elevated Plus Maze
GABA	gamma-aminobutyric acid
GHS-R	growth hormone secretagogue receptor
GGT	Gamma-glutamyltransferase
HPA	Hypothalamic– Pituitary– Adrenal
HPTLC	High performance thin layer liquid chromatography
HPLC	High performance Liquid chromatography
H_2SO_4	Sulfuric acid
5-HT	5 hydroxytryptamine
HGB	Hemoglobin count
IC_{50}	Inhibitory Concenteration 50 %
LYM	Lymphocytes
LDT	Light Dark test
MCV	Mean corpuscular volume
MON	Monocytes
NAc	Nucleus Accubens
NBF	Neutral Buffered Formalin
NEU	Neutrophil (NEU),
NMDA	N-Methyl-D-aspartic acid
NMDAR	N-methyl-D-aspartate receptor
NADH	Nicotinamide Adenine Dinucleotide
NO	Nitric oxide
NPY	Neuropeptide Y
OECD	Organization for economic corporation and development
PFC	Prefrontal cortex
PTZ	Pentylenetetrazole
mRNA	Messenger Ribonucleic acid
PCV	Packed cell volume
POL-6	Polyherbal preparation
PLT	Platelet count
RBC	Red blood cell count

RT-PCR	Real Time Polymerase Chain Reaction
SGOT	Serum glutamic-oxaloacetic transaminase
SGPT	Serum glutamate-pyruvate transaminase
TST	Tail suspension Test
FST	Forced Swim Test
LD_{50}	Lethal Dose 50%
LCMS	Liquid Chromatography-Mass Spectroscopy
TLC	Thin Layer Chromatography
VTA	Ventral Tegmental Area
WBC	White blood cell count
WHO	World Health Organization

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ABSTRACT

Alcohol abuse is a major problem worldwide and it affects people economy and health. There is relapse in alcohol intake due to alcohol withdrawal. Alcohol deprivation effects are the severe symptoms that appear after 24-48 hrs after last alcohol ingestion. The present investigation was designed to explore the protective effect of a prepared polyherbal preparation named POL-6 in ethanol deprivation effects in Wistar rats. POL-6 was prepared by mixing the dried extracts of six plants *Bacopa monnieri, Hypericum perforatum, Centella asiatica, Withania somnifera, Camellia sinesis and Ocimum sanctum* in the proportion 2:1:2:2:1:2 respectively. The ratios of the plants were selected from the literature. POL-6 was standardized according to the Indian Pharmacopeia standardization parameters. Presence of major phytoconstituents in POL-6 was determined by qualitative phytochemical screening and then POL-6 was further subjected to the phytochemical profiling through HPLC, LC-MS and HPTLC. *In-vitro* antioxidant activities of POL-6 were tested through DPPH and nitric oxide assays. Acute toxicity and sub-acute toxicity study of POL-6 was conducted as per OECD 423 guidelines. The effect of POL-6 on the alcohol deprivation effects was tested using two-bottle choice drinking paradigm model giving animals' free choice between alcohol and water for 15 days. Alcohol was withdrawn on 16^{th} day and POL-6 (20, 50 and 100mg/kg, oral), diazepam (2mg/kg) and fluoxetine (30mg/kg) treatment was given on the withdrawal days. Behavioral parameters were tested using EPM, LDT, TST, FST and actophotometer. For studying the effects of POL-6 on ethanol withdrawal seizures a pentylenetetrazole (30 mg/kg) subconvulsive dosage was given in the withdrawal period. On 18^{th} day blood was collected from the retro orbital sinus of the rats and alcohol markers like GGT, SGOT and SGPT etc. were studied. At end of the study animals were sacrificed and brain was isolated for exploring the influences of POL-6 on the mRNA expression of $GABA_A$ and NMDA receptor subunits in amygdala, prefrontal cortex and hippocampus. The results from the present finding showed that POL-6 contains major phytoconstituents like Withferin A, Quercetin, Catechin, Rutin, Caeffic acid and β-sitosterol. *In-vitro* examination confirmed that POL-6 has potent antioxidant activity giving a point of view for its further *in-vivo* studies. Safety studies showed that POL-6 is nontoxic in nature. In-vivo studies showed that POL-6 possess protective effect in alcohol withdrawal anxiety, depression, seizures and locomotor hyperactivity in rats. Gene expression studies on the isolated brain tissues showed that POL-6 normalizes the GABAergic and NMDA transmission in the amygdala, pre-frontal cortex and hippocampus of the rats and inhibits the ethanol withdrawal behaviors. Therefore

we concluded that POL-6 may have therapeutic potential for treating ethanol-type dependence as it suppresses ethanol withdrawal signs and symptoms.

CHAPTER 1

INTRODUCTION

Alcohol is amongst the most broadly utilized and abused drugs adding to an assortment of restorative and financial issues. Alcohol is a psychoactive medication its main impact on the cerebrum is as a focal sensory system depressant [1]. Ceaseless extreme intake of ethanol for extensive periods causes tolerance and reliance on ethanol which may results in organ and tissue damage. Alcohol is legal and socially adequate in many nations and is additionally the most utilized drug around the world [2]. It is consumed predominantly for relaxation, fun and social reasons. Despite the fact that a larger part of individuals consumes alcohol in moderate amounts which can really prompt positive health results, the worldwide burden of illness identified with alcohol consumption is higher [3]. It is for the most part in light of the fact that incautious alcohol consumption isn't connected just with long haul health impacts but instead with intense results, for example, vehicle crashes, drowning or freezing. Such antagonistic results lead to unexpected deaths and inability, particularly among youngsters. Overabundance drinking is likewise connected with diminished profitability, crime, brutality or identity crumbling. Alcohol abuse can prompt alcohol dependence which is a sickness that may include craving, loss of control, tolerance and physical dependence [4]. Chronic consumption of alcohol prompts numerous neuroadaptations and makes an allostatic state- a state in which the chronic presence of alcohol produces a constant challenge to regulatory systems. Modification in brain reward and stress framework occurs because of such changes [5]. These impacts feature themselves through neuroadaptations in hippocampus, amygdala, prefrontal cortex, and numerous different regions. Neurotransmitter systems like dopamine, gamma-aminobutyric acid (GABA), glutamate, serotonin, and a few neuropeptides referred to progress toward becoming deregulated because of ceaseless drinking and alcohol misuse [6].

The alcohol withdrawal syndrome is a state that shows up after decrease or sudden end of consistent heavy drinking in individuals experiencing alcohol dependence [7]. According to the World Health Organization (WHO) report 76.3 million individuals over the world have alcohol use disorders and reliance which results in 1.8 million deaths for each year. Alcohol dependence is characterized by enthusiastic alcohol seeking, unsuccessful in restricting alcohol drinking, and presence of withdrawal syndrome without alcohol [8-9]. Abstinence from chronic alcohol consumption prompts over-excitation of glutamatergic neuronal

synaptic transmission in the amygdala, which results in anxiety characterized by a raised negative emotional response. Negative enthusiastic reactions originating from ethanol withdrawal side effects are the detailed reasons that lead to backsliding to alcohol use. Withdrawal symptoms generally show up inside 6-24 hrs of restraint from alcohol [10]. Manifestations incorporate a rise in blood pressure, anxiety, agitation, hyperreflexia, tremors, queasiness or heaving, sleep deprivation, perspiring, tachycardia, tremors, fractiousness and depression, which may progress to severe conditions, for example, seizures, delirium tremens (DTs), confusion, excessive hallucinations and extreme autonomic instability. Manifestations may prompt serious forms marked by seizures, heart failure and death happen in 5% to 10% of patients [1,11,12]. One of the factors in human drunkards which lead them to heavy drinking is the utilization of alcohol to get calmed of the withdrawal manifestations or to re-experience a similar dimension of the compensating impacts recently experienced with ethanol [13]. Alcohol withdrawal works as an unconditioned stressor for invigorating unconditioned withdrawal reactions, which prompts the actuation of a few cerebrum areas, particularly the regions that are engaged with the tweak and articulation of anxiety and depressive-like behaviors, for example, the amygdala, prefrontal cortex, hypothalamus, and hippocampus. Among the different limbic structures, the amygdala and hypothalamus enactment are believed to have an imperative role in modulating ethanol withdrawal anxiety-like behaviors [14,15]. Ethanol utilization prompts neurobiological and behavioral alterations which are intervened by NMDA (N-Methyl-D-aspartic acid) excitatory and $GABA_A$ (γ-aminobutyric acid) inhibitory receptor frameworks. Ethanol reliance results in a diminished GABA-A neuroreceptor response and up-regulation in excitatory NMDA receptors, this regulate ethanol drinking reinforcement reward, resilience, dependence, and withdrawal. GABAergic and NMDA/glutamatergic frameworks can be the imperative medication focuses on accomplishing a long haul forbearance from alcohol [1-2]. At present treatment choices for alcohol withdrawal syndrome are not many, for example, Benzodiazepines, Disulfiram, Naltrexone and Acamprosate. Benzodiazepines are the main medication among all others, yet their utilization is related to different symptoms, for example, sedation, psychological hindrance and has addictive properties which make a constraint to their use. Subsequently, the assurance of new powerful and therapeutically beneficial medications for the treatment of alcohol withdrawal indications is imperative [16,17].

Plants are the important sources of medication and a large number of drugs being used are derived from plants. Viability of restorative plants in the diseases management is apparent, and the World Health Organization has perceived its utilization in essential medicinal

services conveyance framework [18]. Herbal drugs are perceived as the significant source of therapeutics and are the principal choices in medicinal services treatment these days as they are better compatible with the human body and have insignificant reactions and desirable results. In view of a blend of conventional utilization and continuous scientific research, the capability of plant-based preparations for the aversion of sicknesses and improvement in wellbeing quality has pulled in much research consideration [19]. Polyherbal preparations have accomplished wide acknowledgment in contrast with unrefined plant extracts and are broadly utilized for treating different disorders because of convenience, decrease in dose proportion and simplicity of administration. Polyherbal preparations are well known worldwide as restorative operators, in different ailments that sway the quality of life. A large portion of the synthetic medications give symptomatic alleviation by following up on a solitary molecular target although the multi target action of Polyherbal preparations are helpful in interminable conditions, for example, cancer, diabetes, osteoporosis, etc. It ought to be noticed that herbal preparations have been esteemed for their additional viability because of the synergistic impact of numerous herbs [20-22].

Hypericum perforatum Linn (St. John's wort), family Hypericaceae is accounted for to have anti-anxiety, antidepressant, antidiabetic, pain relieving and other mood disorders related properties. The major active constituents in *Hypericum perforatum* are hypericin, hyperforin, quercetin, kaempferol, pseudohypericin, luteolin, rutin, isohypericin, and hyperoside [23]. *Bacopa monnieri* (L.) Pennell usually known as Brahmi family Scrophulariaceae is accounted for to have defensive impacts against neurological disorders like epilepsy, depression, stress, insanity, psychosis, and sleep deprivation. The chief components in *Bacopa monnieri* include brahmine, bacoside A and B, bacopasaponins A, B and C, herpestine and D-mannitol [24]. *Centella asiatica* (L.) Urban (Gotu kola) family Apiaceae is accounted for to have different pharmacological activities like an antiepileptic, antidepressant, nervine tonic, rejuvenant, sedative and tranquilizer. *Centella asiatica* contains phytoconstituents like asiaticosides, madecassoside, Asiatic acid, pentacyclic triterpenoid saponins, madasiatic acid, campesterol, and sitosterol [25]. *Withania somnifera* (L.) Dunal usually known as Ashwagandha family Solanaceae is professed to have antianxiety, pain relieving, antistress and anti-inflammatory properties. *Withania somnifera* contains phytoconstituents like withaferins, withanolides, withananine, sitoindoside, isopellertierine, somniferine, somnine and tropine [26]. *Ocimum sanctum* Linn. commonly known as Tulsi family Lamiaceae have pain relieving, antistress, antipyretic, anticonvulsant, neuroprotective, immunomodulatory, calming, and memory enhancer properties. *Oscimum sanctum* has active phytoconstituents like euginal, eugenol,

carvacrol, urosolic acid, rosmarinic acid, apigenin and caryophyllene [27]. *Camellia sinensis* (L.) Kuntze commonly known as Green tea family Theaceae have pharmacological properties like anti-Parkinson, anti-aging, antistroke and anti-Alzheimer's. The active phytoconstituents present in *Camellia sinensis* are gallocatechin, catechin, gallocatechingallate, quercetin, catechingallate, myricetin, and kaempferol [28]. These plants have different pharmacological properties and have been utilized for the preparation of polyherbal preparations in the Ayurvedic system of drugs for treating many ailments. In previous investigations, it has been discovered that a blend of *Camellia sinensis*, *Bacopa monnieri* and *Hypericum perforatum* have synergistic antioxidant activities [29]. Owing to the fact of synergistic activities and diverse pharmacological effectiveness of these medicinal plants, in the present study a polyherbal preparation (POL-6) comprising six plant extracts *Withania somnifera*, *Camellia sinesis*, *Hypericum perforatum*, *Centella asiatica, Bacopa monnieri* and *Ocimum sanctum* was developed, standardized and pharmacologically evaluated in the alcohol deprivation effects followed by voluntary alcohol consumption in rats. For the development of POL-6 most effective doses of the plants were selected from the literature and POL-6 was prepared by mixing all the plant extracts.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Alcoholism

Alcohol addiction is a chronic ailment with hereditary, psychological and environmental factors influencing its development. Alcohol misuse implies drinking more alcohol than is socially, mentally or physically worthy. Alcohol is doubtlessly among the most reliance instigating medications of abuse. The most established and most broadly utilized drug on earth is alcohol [2,30]. The drinking of beverages containing ethyl alcohol influences physiological and mental impacts [3,31]. Alcohol is additionally expended even as a piece of religious practices and inside a particular social setting. The expanded danger of alcohol abuse, lack of healthy sustenance, interminable pancreatitis, alcoholic liver disease, and cancer are related in view of elevated amounts of alcohol utilization with extra harm to the central nervous system and peripheral nervous system can happen from long term alcohol utilization [32]. The dangerous impacts of ethanol are especially vulnerable to the developing adolescent brain moreover, the creating fetal brain is additionally vulnerable, and if a pregnant woman consumes alcohol fetal alcohol syndrome may occur. The alcoholism can change the cerebrum subsequently and it is viewed as a brain ailment as it changes its structure and how it functions. It might result in durable cerebrum changes and can prompt the unsafe behaviors found in individuals who abuse alcohol [33]. As per WHO report, around 2 billion people devour ethanol and 75.9 million have ethanol use disorder [8,34]. A similar report gauges 1.8 million yearly deaths (3.2% of aggregate) and 58.3 million (4% of aggregate) of disability-adjusted life years (DALYs) credited to alcohol globally. Alcohol establishes the biggest hazard factor for DALYs lost in middle-income nations and the third biggest in all income group nations. Studies have uncovered that alcohol utilization is related to in excess of 60 kinds of ailment and injuries [35,36]. WHO 2005 report ascribed 31·7% of all year's lived-with-inability to neuropsychiatric conditions: the main issue that added to this all out was unipolar major depression (11·8%) trailed by alcohol use disorder (3·3%). Be that as it may, unrecorded alcohol utilization is evaluated to be something like 66% of all alcohol utilization in the Indian subcontinent, about half of utilization in Africa, and around 33% in Eastern Europe and Latin America. Other than devastating medical and psychiatric

consequences for the alcoholic, alcohol enslavement is a heavy burden to family, companions and social services and is a huge part in wrongdoing and car crashes [8,36,37].

2.2 Pharmacokinetics of alcohol

Alcohol absorption starts in stomach (20%) and around 80% in the small intestine. Through the portal vein, alcohol reaches in the liver where it is metabolized (Fig. 2.1). Alcohol is metabolized to acetaldehyde either by cytochrome P450 or alcohol dehydrogenase. The acetaldehyde at that point is oxidized to acidic acid and water. Alcohol digestion by alcohol dehydrogenase results in increased NADH (Nicotinamide Adenine Dinucleotide).

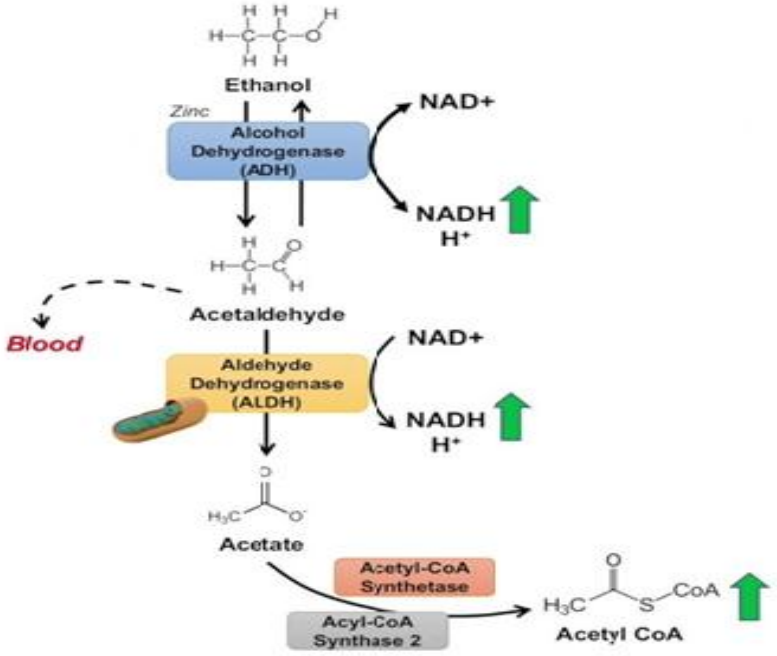


Figure 2.1: Alcohol Metabolism.

Over the top NADH levels can restrain gluconeogenesis and oxidation of fat molecules. Alcohol is a CNS depressant that shares numerous pharmacological properties with non-benzodiazepines sedative-hypnotic medications. It influences the CNS in a dose-dependent manner, delivering sedation that advances to sleep, surgical anesthesia, unconsciousness, coma, respiratory melancholy and cardiovascular collapse. Alcohol intake results in an expansion in endogenous opioids and this is in charge of euphoric impact on alcohol supposition. As of now, there are no clinically helpful opponents that turn around the pharmacologic impacts of alcohol. The lethal dose of alcohol in people is variable, yet demise happens when blood levels are 400-700 mg/dl [38-40].

2.3 Impacts of alcohol on the body

Numerous organs in the body could be influenced by alcohol utilization (Fig. 2.2). It might make damage and side effects to the body organs based on expansive and little alcohol utilization. Chronic alcohol utilization prompts pancreatitis, gastritis, lack of healthy sustenance, and cirrhosis of the liver. Hypertension, high-fat dimensions in the blood, rhythm aggravations and congestive heart failure can be caused as an inordinate utilization of alcohol. Depression, memory impairment, emotional and sleep issue can be the impact of unending alcohol ingestion on the brain. Feebleness and sterility could be caused because of prolonged alcohol utilization. Psychomotor execution and obtuse reflux motor activity debilitated due to alcohol utilization. The danger of hepatic, pancreatic and esophageal malignancy is expanded because of endless alcohol ingestion and can prompt fetal alcohol syndrome amid pregnancy, consequently is teratogenic [41,42].

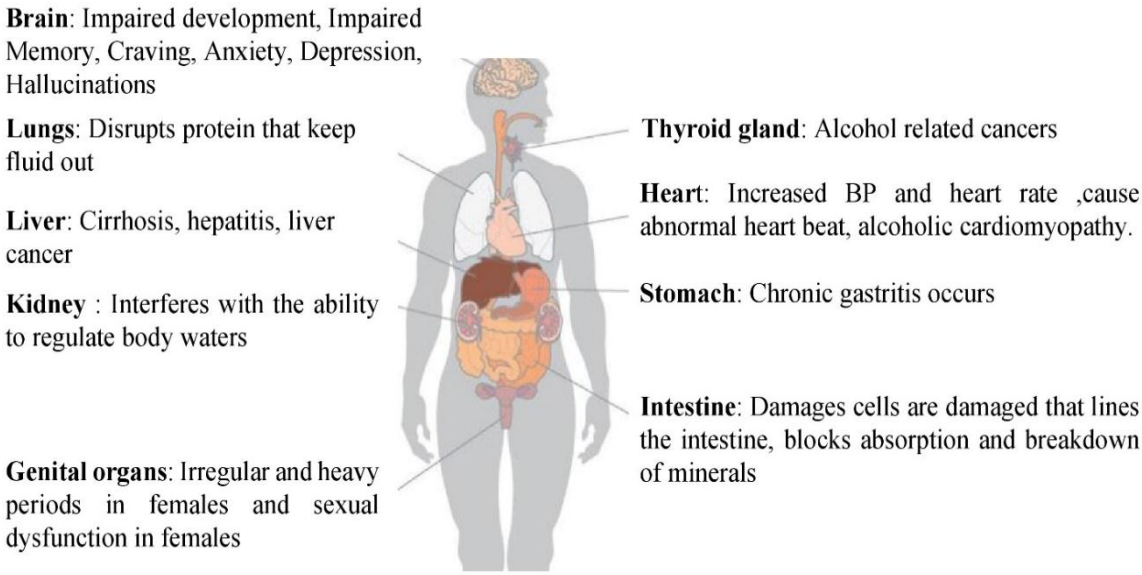


Figure 2.2: Alcohol effects on the body.

2.4 Blood alcohol concentration (BAC)

The concentration of ethanol in the blood is known as BAC. It is estimated either as by mass per volume or a percentage by mass. In the United Kingdom, BAC is accounted for as milligrams of ethanol per 100 milliliters of blood. In many nations, BAC is estimated and revealed as grams of ethanol per 1,000 milliliters of blood. For motivations behind law authorization, BAC is utilized to characterize inebriation and gives a rough proportion of hindrance. Most nations forbid operation of watercraft, vehicles, heavy machinery and airship

above recommended dimensions of BAC. BAC is normally alluded to as blood ethanol content [43,44].

2.5 Alcohol abstinence syndrome

Alcohol abstinence syndrome is the set of manifestations seen when a person diminishes or stops ethanol utilization after perpetuate times of redundant ethanol consumption. Overconsumption of ethanol prompts physical reliance, tolerance and alcohol abstinence syndrome [7,45]. The abstinence syndrome is to a great extent because of the central nervous system being in a hyper-excitable state. In contrast to most withdrawals from different medications, alcohol withdrawal can be lethal. The abstinence syndrome can incorporate convulsions and delirium tremens and may prompt excitoneurotoxicity [46]. Alcohol withdrawal is described by disabled physiological capacity and improved negative effect. Physical withdrawal side effects incorporate aggravated sleep patterns, seizures, tremor, perspiration, queasiness, and regurgitating. The negative effect in withdrawing heavy drinkers incorporates depressed mood and anxiety, both of which have been related to relapse. It has been recommended that the negative fortification in diminishing the negative influence related to withdrawal symptoms is a noteworthy contributing element in a relapse in heavy drinkers [2,14]. All the more explicitly, expanded uneasiness and feelings of anxiety appear to be firmly linked with expanded alcohol consumption. Alcohol dependence and major depressive like behavior are firmly associated, with people who experience both major depressive episodes and alcohol dependence [47]. It has likewise been discovered that different sorts of anxiety disorders, for example, agoraphobia, social dread, panic disorder, simple phobia and post-traumatic stress disorder are emphatically related with expanded alcohol consumption. An early manifestation of alcohol withdrawal is anxiety [48]. It is suspected that early withdrawal anxiety is a critical factor in the chronic usage of alcohol in alcohol-dependent people. In people, physical withdrawal symptoms keep going for 12-72 hours after the last intake, yet abstinent heavy drinkers report craving for quite a long time after withdrawal. Alcohol withdrawal symptoms are categorized based on the timespan of the last drink of alcohol consumed i.e. 6-12 hrs. (shaking, cerebral pain, perspiring, anxiety, nausea or spewing), 12-24 hrs. (confusion, hallucinations (with attention to the real world), tremor, agitation, and comparable infirmities), 24-48 hrs. (seizures), >48 hrs. (delirium tremens, portrayed by illusions that are indistinct from the real world, convulsions, severe

confusion, fever and hypertension which can endure somewhere in the range of 4 to 12 days) [10,49].

2.6 Pathophysiology

Ceaseless utilization of alcohol prompts alterations in central nervous system, particularly in the GABA framework. Different adaptations happen, for example, changes in mRNA expression and down-regulation of $GABA_A$ receptors. Amid intense ethanol withdrawal, alterations likewise happen, for example, down-regulation of α1 and α3 comprising $GABA_A$ receptors. Neurochemical alterations happening amid ethanol withdrawal can be constrained with medications that are utilized for intense detoxification [50]. With forbearance from ethanol and cross tolerant medications these progressions in neurotransmitters step by step becomes ordinary. Adjustments to the NMDA framework additionally happen because of rehashed alcohol inebriation and are engaged with the over excitation of the CNS amid the ethanol abstinence syndrome [2,51]. The increased levels of homocysteine amid perpetual drinking increment significantly further amid the abstinence state and results in energizing neurotoxicity. EEG abnormalities may happen amid early withdrawal like alterations in ECG, specifically an expansion in QT interval and dysfunction of the HPA (Hypothalamic Pituitary Adrenal) axis and expanded discharge of corticotrophin-releasing hormone happens amid both acute and extended restraints from ethanol and add to acute and extended abstinence manifestations [2,46]. Anhedonia/dysphonia manifestations, that can endure as a component of an extended withdrawal might be because of dopamine under activity. Alcohol withdrawal syndrome is described by hyperactivity of the nervous system. This hyperactivity represents the cerebrum's endeavor to work ordinarily in spite of the inhibitory impact of endless alcohol utilization. The syndrome shows when alcohol utilization stops [1,52].

2.7 Neurobiology behind alcohol withdrawal syndrome

Alcohol has specific effects on certain receptors and neurotransmitters in the brain. Alcohol interrupts the normal functioning of neurotransmitters, and can prevent the neurotransmission that would normally affect certain types of behavior [54].

2.7.1 Gamma-aminobutyric acid

GABA is the primary inhibitory synapse in the mammalian focal sensory system. It follows up on three classes, named as $GABA_A$, $GABA_B$ and $GABA_C$. The $GABA_A$ receptors are

ionotropic and found in the vast majority brain neurons and spinal cord and intercede significant CNS depressant impacts. GABA works by connecting to a coupling site on $GABA_A$ receptors, making a pore in the cell membrane to open and admit chloride ions. The stream of these negatively charged ions into the neuron renders it less sensitive to encourage neurotransmission. Ethanol-intervened enhancement of GABA activity is believed to add to the intense anxiolytic and sleep inducing impacts of alcohol [55,56]. More straightforward indication shows that the GABA framework modulates ethanol utilization. For instance, animal studies showed that $GABA_A$ receptor antagonists, for the most part, diminish alcohol self-administration. Then again, treatment with a neuroactive agent that improves $GABA_A$ receptor activity expanded ethanol admission. These findings recommend that alcohol-interceded improvement of $GABA_A$ receptor activity or GABA discharge advances ethanol utilization. $GABA_A$ receptors have been embroiled in an assortment of conditions, including anxiety, stress, epilepsy, depression, sleep deprivation, and learning and memory; moreover, they add to different intense impacts of alcohol, for example, sedation and anxiolysis. The action of GABA on $GABA_A$ receptors is further enhanced by sedative agents, such as benzodiazepines, barbiturates, and general anesthetics, which do not bind to the same site on the receptor as GABA but act at different sites [57,58]. $GABA_A$ receptors—with restricting locales for different molecules and the chloride ion channel form buildings of five protein subunits (α, β, γ, δ, ε, θ and π) and there is more than one kind of subunit for few other categories (α1-6 and β1-3). The particular structure of receptor molecule decides its different biological and pharmacological activities [59,60]. Initiation of synaptic $GABA_A$ receptors is reliant on GABA discharge at the neurotransmitter and may result in a momentary inhibitory impact (known as a phasic hindrance). Enactment of extra synaptic $GABA_A$ receptors assumes a role in creating a stable electrical flow that is available in neurons at their resting potential and isn't subject to synaptic GABA discharge (known as a tonic hindrance). Numerous examinations have explored the alcohol association with $GABA_A$ receptors. By and large, these investigations confirmed that intense alcohol consumption improves GABAergic neurotransmission. In any case, electrophysiological examinations of alcohol's impacts on $GABA_A$ receptor activity rarely yield any predictable outcomes. In a few cases like a particular test temperature were expected to show any impacts, which brought up issues with respect to the physiological significance of these impacts [61,62]. In various cells the inconstancy of ethanol influences outcomes from contrasts in the organization of the $GABA_A$ receptors. For instance, δ subunit receptors might be utmost delicate to alcohol-prompted increase in function. After constant ethanol exposure the intense impacts of alcohol

diminished GABA neurotransmission as an adjustment to steady initiation by ethanol. This reduced inhibitory activity trigger anxiety and neuronal hyper excitability saw amid intense alcohol withdrawal [63]. A later report utilizing perpetual irregular alcohol exposure (i.e., a few episodes of ethanol introduction and withdrawal), be that as it may, revealed impaired $GABA_A$ receptor function in the hippocampus; additionally, the animals displayed more prominent susceptibility to seizures and expanded anxiety. Reduced activity of $GABA_A$ receptors may contribute to the efficacy of benzodiazepines, which potentiate the activity of many subtypes of $GABA_A$ receptors, in controlling seizures and convulsions induced by alcohol withdrawal. However, chronic alcohol consumption has the opposite effect on the activity of GABA neurons in the VTA as on GABA systems in other brain areas, the VTA neurons show increased activity. This increase may reflect the increased glutamatergic activity that occurs during alcohol withdrawal (Fig. 2.3). This increased GABA activity would contribute to the reduced mesolimbic dopamine release associated with withdrawal [61,64,65].

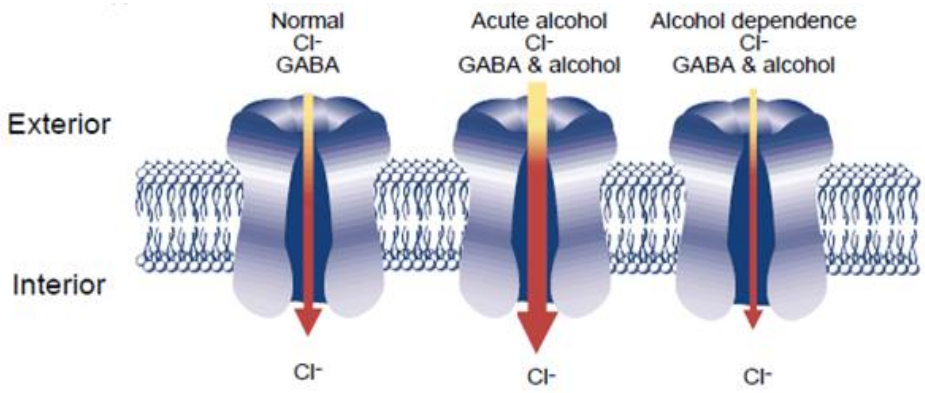


Figure 2.3: $GABA_A$ receptor sensitivity is reduced. GABAergic inhibitory neurotransmission is decreased after ceaseless alcohol consumption. Without alcohol, the synapse GABA opens $GABA_A$ receptor-chloride (Cl-) channels, repressing neurotransmission. Alcohol builds GABA's impact, enabling chloride to enter the neuron in a greater extent and expanding inhibition. In alcohol reliance, both GABA and ethanol have little impacts on $GABA_A$ receptors. Low chloride expands the enactment of neurons already hindered due to GABA neurotransmission [46].

2.7.2 Glutamate

Glutamate follows up ionotropic and metabotropic receptors. The ionotropic receptors are grouped into NMDA, Kainate and AMPA receptors and are ligand-gated ion channel receptors. The metabotropic receptors are isolated into eight mGluR subtypes and are G-protein coupled receptors. Approximately 40% of all neural connections in the brain are

believed to be glutamatergic, so glutamate is viewed as a noteworthy excitatory synapse. Glutamate seems to have a basic job in memory and cognition [66,67]. Ethanol, at pharmacologically applicable concentration, represses glutamatergic neurotransmission, essentially by following up on iGluRs, albeit a few impacts likewise have been noted on mGluRs. Introductory reports exhibited that intense alcohol exposure hinders NMDAR channel activity in cerebellum and hippocampal segregated neurons and in numerous different frameworks, like the cerebral cortex, VTA and amygdala. These examinations showed that inhibition of NMDAR actuation by alcohol is noncompetitive with glutamate and the alcohol doesn't dislodge glutamate from the NMDAR; rather, receptor enactment is decreased. Alcohol additionally restrains AMPAR carriers by a noncompetitive mechanism [68,69]. Since the deluge of cations by iGluRs amid excitatory synaptic transmission is basic for instigating versatility, the intense alcohol exposure contrarily influences the acceptance of NMDA-subordinate protracted haul potentiation just as advances protracted haul depression. Not all iGluRs give off an impression of being similarly sensitive to intense ethanol exposure. AMPARs display a huge contrast in ethanol sensitivity that is subunit composition subordinate. Along these lines, AMPARs involving both GluR2 and GluR3 subunits, and receptors including just GluR3 subunits, were less delicate to inhibition by ethanol than every single other combination tried. A large number of the behavioral impacts of intense ethanol exposure can be connected to consequences for glutamatergic neurotransmission. Pharmacological agents that, similar to ethanol, inhibit iGluRs activity have ethanol-like discriminative boost properties in rodents and, now and again, make the animals considerably progressively sensitive to the locomotor stimulant impacts of low dosages of ethanol. So also, in detoxified alcohol subordinate people, NMDAR antagonists, for example, ketamine produce subjective inebriating impacts that take after those of alcohol [70,71]. In animal models, treatment with mGluR5 inhibitors diminished the compensating impacts of alcohol under certain test conditions, diminished alcohol consumption, and forestalled alcohol subordinate changes in glutamate and dopamine discharge from NAc neurons. Intense ethanol exposure additionally displays presynaptic impacts on glutamatergic signal transmission. In spinal motor neurons of infant rodents, ethanol diminished the recurrence of NMDAR-and AMPAR-subordinate postsynaptic electrical signs (purported excitatory postsynaptic currents [mEPSCs]), recommending that ethanol repressed glutamate discharge into the neural connection. So also, intense ethanol exposure decreased the recurrence and abundance of NMDA-interceded mEPSCs in neurons in the NAc. Such impacts might be interceded by ethanol-sensitive mGluRs on presynaptic axon terminals [69,72,73]. Different

examinations found that when presynaptic mGluR2/3 were hindered, the intense sedative and hypnotic inducing impacts of ethanol in mice were decreased. This finding proposes that ethanol advances the initiation of these mGluRs. At the point when glutamate receptors are inhibited for broadened timeframes as a result of supported ethanol exposure, the body attempts to adjust to the perpetual presence of ethanol and utilizes a few mechanisms to keep up ―normal receptor activity even within the sight of ethanol [71]. For instance, after long haul ethanol exposure, when ethanol has been eliminated from the cells, the capacity of NMDARs in cortex and cerebellum is observed to be expanded. In addition, after endless alcohol exposure in rodents, the creation of NMDAR subunits was seen expanded in amygdala, cerebral cortex and hippocampus. Because of its consistent restraint of glutamate transmission, constant alcohol consumption in rodents prompts a compensatory NMDA receptors up regulation [74,75]. Consequently, ethanol abstinence is related with expanded excitatory amino acid communication and shows into manifestations, for example, seizures. Withdrawal after perpetual ethanol treatment likewise evoked drawn out and intemperate NMDAR-subordinate movement in specific neurons (i.e., CA1 pyramidal neurons) detached from rodent hippocampus that is like the action seen amid epileptic seizures.

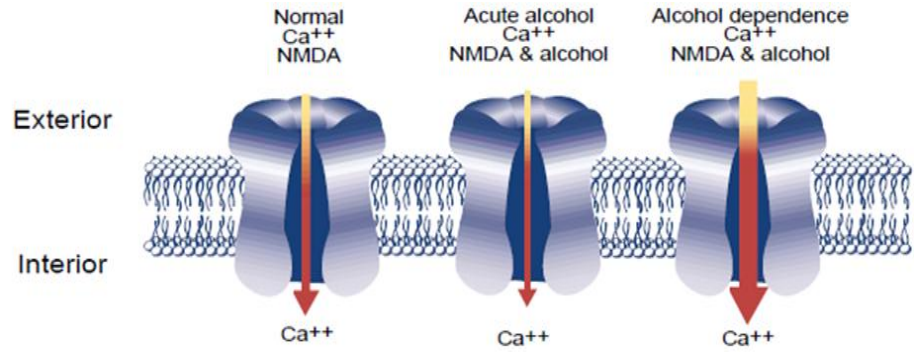


Figure 2.4: NMDA receptor sensitivity is enhanced. Glutamatergic excitatory neurotransmission builds following constant ethanol consumption. Without the alcohol the synapse glutamate enables the entry of calcium to axon by means of the NMDA receptor carriers. Transient ethanol exposure diminishes calcium stream into the axon by the receptor channels. In ethanol reliance, NMDA receptor delicacy to glutamate increments bringing about overabundance stream of calcium into neurons prompting hyper excitation [46].

The ethanol withdrawal– incited hyper excitability inclines neurons to excitotoxic cell death if the NMDARs are stimulated. Compounds that go about as NMDAR antagonists, including MK-801 and ifenprodil, can ensure the cells against withdrawal-incited neurotoxicity (Fig. 2.4). The antagonists of mGluR5 were likewise observed to be helpful in averting alcohol backslide [46,69,76].

2.7.3 Glycine receptors

Glycine receptors are ligand-gated chloride channels which assume a noteworthy role in inhibitory activity in the developing central nervous system, especially in the spinal cord and medulla. The α and β subunits are arranged on the postsynaptic layer in pentameric structure and repress axonal firing by opening the Cl^- carriers ensuing agonist binding. Alcohol goes about as an allosteric modulator of the $GABA_A$ receptor complex and potentiates the GABA gated current further expanding the hyperpolarization [77]. This potentiation was found to be because of an expansion in the number of channel openings and bursts just as the term of channel openings. The alcohol initiated conduct changes following intense consumption are upgraded by GABA agonists and antagonized by GABA antagonists. Ion transition contemplates performed in synaptoneurosomes have demonstrated that alcohol additionally potentiates the inhibitory glycine receptors. Ro-15-4513, a negative allosteric modulator of the $GABA_A$ receptor represses the potentiating impacts of alcohol in concentrates with recombinant receptors communicated in Xenopus oocytes [46,78]. The medication has likewise been appeared to reverse the impacts of ethanol inebriation in rodents. Anyway, its anxiogenic and proconvulsant action kept Ro-15-4513 from being tried for clinical trials in human. A region of 45 amino-acid deposits has been recognized utilizing chimeric receptor builds inside $GABA_A$ and glycine receptors as the putative alcohol binding site. Inside this locale, two explicit amino-acid deposits in trans membrane domains 2 and 3 were observed to be critical for allosteric regulation of the receptors [77]. The Glycine receptors likewise impact extracellular dopamine levels and alcohol prompted dopamine discharge in the NAc which is a critical part of the cerebrum reward circuit. An examination led in male grown-up Wistar rodents demonstrated that reverse micro dialysis of glycine into the NAc diminishes alcohol consumption. Org25935, a glycine reuptake inhibitor, was likewise appeared to lessen alcohol consumption in alcohol-preferring Wistar rodents by expanding extracellular glycine levels and in this way tweaking brain glycine receptors [77,79].

2.7.4 Dopamine

Dopamine synthesis as well as discharge increases following intense utilization of alcohol. Alcohol utilization has been appeared to induce the release of dopamine in the NAc, a noteworthy component of brain reward pathway. The barricade of dopamine receptors in the NAc decreased alcohol consumption. Deprivation from alcohol prompts mesolimbic dopamine framework hypo function and it is assumed that this abatement in function

motivates the person to continue alcohol consumption. Studies utilizing rodent models of ceaseless alcohol utilization demonstrated underlying mechanisms of dopamine framework hypo function to be over activity of calcium channels and a decline in tyrosine hydroxylase. In a clinical investigation of abstinent alcoholic people, lower accessibility of dopamine receptors in the ventral striatum and adjoining putamen was associated with higher alcohol craving behavior. Subsequently, the neuroadaptive changes in the mesolimbic dopamine framework assume a critical role in negative strengthening impacts of alcohol in alcoholism [80-83].

2.7.5 Opioid receptors

There are three types (μ, δ and κ) of opioid receptors which are the objectives for endogenous opioid peptides, for example, β-endorphin, enkephalins, and dynorphins. Endogenous opioids assume a noteworthy role in the cerebrum reward framework through the modulation of the mesolimbic dopaminergic framework. Alcohol meddles with the opioid mechanisms both at the dimension of the endogenous opioid peptides by influencing their synthesis, discharge, or processing and at the dimension of the opioid receptors by changing their proclivity for the opioid peptides. Intense exposure to alcohol has been appeared to build enkephalin and β-endorphin levels in the cerebrum and higher β-endorphin levels have been connected to alcohol addiction. Opioid antagonists that are particular for μ-and δ-narcotic receptors, just as some nonselective antagonists, for example, Naltrexone and Naloxone have been accounted for to diminish alcohol consumption. Their mechanism of action, be that as it may, is disagreeable. The opioid antagonists were thought to act by hindering alcohol actuated dopamine discharge in the NAc. In any case, in lesion investigations of dopaminergic terminals in the NAc, while the lesions couldn't adjust alcohol consumption, Naltrexone had the capacity to decrease the intake. Henceforth the impacts of opiate antagonists can't be completely ascribed to their actions on dopaminergic transmission. As alcohol increments extracellular endorphins in the NAc, the opioid antagonists are likewise thought to act by restraining endogenous endorphin activities [83-87].

2.7.6 Nicotinic acetylcholine receptors

Nicotinic acetylcholine receptors (nAChRs) are present in the peripheral nervous system and cerebrum. It was recommended that alcohol's impacts on nAChRs could be incharge of its addictive nature. A study showed that cholinesterase inhibitors decreased alcohol utilization

in alcohol-preferring rodents, but it isn't clear concerning which subunit arrangement assumes a noteworthy role in this action [84,88,89].

2.8 Bain reward system and alcohol withdrawal

A few neuroanatomical components and synapses are suggested in reward, with the mesolimbic dopamine framework being simply the most sensitive to electrical incitement. Meeting proof from self-administration, pharmacological, physiological and behavior studies point to the mesolimbic dopamine framework as the center substrate for reward and positive reinforcement, the VTA is situated in the ventral midbrain medial to the substantia nigra and includes dopamine neurons that venture through the medial forebrain pack to the limbic structures nAc, amygdala and hippocampus and to the medial prefrontal cortex [90-92]. The VTA, nAc pathway is known to be center for mediating the act the reward stimulus as well as reinforcement and motivation for reward oriented behavior. Alcohol affects the glutamine, GABA, dopamine, and endogenous opioid systems. Alcohol consumption is associated with a release of β-endorphins, produced in the arcuate nucleus, which stimulate dopamine release in the nucleus accumbens, both directly and indirectly by inhibiting GABA production in the VTA. The release of β-endorphins, coupled with the release of dopamine, is thought to result in the pleasurable feelings associated with alcohol dependence. The reward is related to an event for which an animal will perform on operant reaction. Outer and inward stimuli that are capable of expanding the recurrence of the operant reaction are named as reinforces; and stimuli that decline the recurrence named as punishers. Substances of abuse are self-administered because of their positive strengthening impact, which is to avoid undesirable withdrawal manifestations [94]. The primary locales in the cerebrum that intercede alcohol reinforcement are the nucleus accumbens (NAc), VTA and the PFC which comprise the mesocorticolimbic dopamine framework and the extended amygdala. On accepting the stimulus that is reinforced, the limbic framework forms all the new and recently learned data related to it. The limbic structures engaged with this function incorporate the basal horizontal amygdale which offshoots emotional processing and the HPC which makes relevant affiliations. The job of the PFC is the assurance of objective oriented activity. The signs from the PFC and limbic structures enact the nucleus accumbens, which verbalizes the received data and transfers it to the motor framework to create the motor activities identified with the reaction. The NAc does as such by means of disinhibiting substantia nigra reticulate and motor thalamus, further actuation of motor cortex which produces motor reaction through its

projections to the spinal cord. There are solid dopaminergic projections from VTA to NAc and furthermore to the PFC [93]. Alcohols increment the firing of these neurons, which lead to increment in extracellular dimensions of dopamine in the NAc. In addition, GABAergic inhibitory projections arising in the NAc project to the ventral pallidum which further projects to VTA and the dorsal medial thalamus (DMT). The DMT sends glutamatergic efferent to PFC finishing the limbic circle. Further, the efferent between AMG and PFC and from the PFC to the NAc are glutamatergic, the PFC likewise sends a solid glutamatergic efferent to the dopaminergic neurons in the VTA. Enactment of these neurons has been legitimately connected to addictive behavior. Aggregation of extracellular glutamate because of alcohol withdrawal has been seen in the NAc, striatum, and HPCs [95,96].

2.9 Diagnostic criteria for ethanol abstinence

1. Discontinuance or decrease in ethanol intake which has been prolonged or substantial. 2. At least two of the given side effects seen inside hours to a couple of days after precedent 1, Increased autonomic activity (perspiring or more than 100 beats/minute), sleeplessness, acoustic illusions, spewing, cognitive impairment, convulsions and anxiety. 3. The side effects in precedent 2 cause huge impairment in social, work related, or another important operational area. 4. The manifestations not owing to a normal medical situation and are worse represented by another psychological disorder [97,98].

2.10 Alcohol withdrawal anxiety

Alcohol withdrawal anxiety-related behavior is showed by outrageous dread and tension, joined by heart pulsation, panting, perspiring and dazedness. Ethanol shows antianxiety effects which elevate its utilization. Be that as it may, delayed ethanol use and especially extreme alcohol withdrawal conditions may expand anxiety levels. Stamped signs of anxiety usually show up in the range of 12 to 48 hours after abstinence from ethanol consumption [1,10].

2.11 Alcohol withdrawal depression

Depressive manifestations generally seen in alcoholics who are inebriated or experiencing ethanol detoxification. This finding might be inferable from the arrival of social restraint-related with alcohol inebriation or with the depressive inclination expresses that go with the decay from pinnacle inebriation. Depressive disorders regularly rise amid alcohol

withdrawal; notwithstanding the depressive inclination conditions related with ethanol utilization and abstinence, mental, physical and social issues related with alcohol addiction add to the advancement of depressive like behavior [10,33].

2.12 Alcohol withdrawal related seizures

This incorporates epileptic form seizures generally grand mal which normally occur within 12 to 48 hours of ethanol suspension and may occur before the BAC has tumbled to zero. The prevalence of alcohol withdrawal seizures is assessed at somewhere in the range of 2 and 9% of alcohol subordinate people. The danger of seizure repeat inside 6-12 hours is evaluated at somewhere in the range of 13% and 24% in untreated patients. After the person stops drinking 90% of ethanol withdrawal related seizures happen inside 48 hours. After last ethanol intake 3% of convulsions may occur between 5 to 20 days. The relationship between the quantity of alcohol detoxification and the development of alcohol abstinence complications, including convulsions, has been ascribed to cumulative long-term changes in brain excitability. It has been proposed that long term neurotoxic impacts of alcohol may prompt epilepsy [99,100].

2.13 Current pharmacotherapy for alcohol dependence and withdrawal

The current accessible treatment for alcohol dependence and withdrawal incorporates drugs focusing on a few distinct frameworks as of non-explicit nature of ethanol activity in the body.

2.13.1 Benzodiazepines

Benzodiazepines are utilized for psychomotor unsettling that people experience amid withdrawal. Diazepam, lorazepam, and chlordiazepoxide are used as often as possible to treat or counteract alcohol withdrawal, yet other benzodiazepines might be utilized. When all is said in done diazepam or chlordiazepoxide favored as they appear to result in a smoother clinical response with a low potential to produce physical dependence and tolerance in short-course therapy. Lorazepam or oxazepam generally preferred for the treatment of patients with cutting edge cirrhosis or intense alcoholic hepatitis. The shorter half-life of lorazepam and the absence of dynamic metabolites with oxazepam may anticipate delayed impacts if over sedation happens. Paradoxically, chlordiazepoxide has a generally long half-life and may prompt over-sedation in patients with the serious liver malady. Parenteral treatment with (e.g.

lorazepam, diazepam) may be required in patients who can't receive oral drugs. Benzodiazepines show their effect by means of incitement of GABA receptors, causing abatement in neuronal movement and relative sedation. When physical reliance on benzodiazepines has created, stoppage of therapy will be joined by withdrawal side effects comprise of tremor, stomach spasms, heaving, perspiring, migraine, muscle torment, anxiety, strain, irritability, and confusion [101-103].

2.13.2 Baclofen

Baclofen is a $GABA_B$ receptor agonist which ended up being beneficial in keeping up alcohol restraint and forestalling relapse in some fundamental clinical examinations. Baclofen is thought to act by inhibiting the $GABA_B$ receptors that are available on cell assemblages of dopaminergic neurons in VTA that project to limbic areas, thereby lessening alcohol initiated dopamine discharge in the limbic framework. The accessible pharmacotherapy for alcohol reliance incorporates drugs acting on alcohol metabolism as well as a wide range of synapse frameworks including endogenous opioid framework, glutamate framework, dopamine framework, serotonin framework, and GABA frameworks [104,105].

2.13.3 Acamprosate

Acamprosate is a FDA-affirmed medication for counteractive action of ethanol relapse. It is a manufactured GABA analog. It has been appeared to connect with presynaptic $GABA_B$ receptors, expanding the release of GABA from presynaptic terminals. It is a homotaurine subsidiary without ethanol-like behavioral impacts that decreases alcohol utilization in animals. It is believed to be a practical glutamate antagonist, which controls the hyper glutamatergic state created by interminable alcohol utilization. It decreases NMDA receptor function, adding to its ability to suppress ethanol withdrawal. It seems to repress calcium ion influx through voltage-subordinate calcium channels and NMDA receptors [106,107].

2.13.4 Naltrexone and other Opioid Antagonist

Naltrexone and other Opioid Antagonist has been depicted as an anti-craving medicine because clinical trials have demonstrated that it calms the urge that helps relieve the urge that alcoholics have to consume ethanol and have been appeared to be effective in bringing down alcohol craving, expanding time to initially relapse and bringing down overall rates of relapse in overwhelming alcohol drinking. This investigation recommends that the opioid antagonists may act by hindering alcohol incited dopamine discharge, in this way lessening the rewarding

impact on alcohol. The mechanism, be that as it may, does not completely clarify their impact on alcohol utilization. Several clinical studies show that naltrexone was not beneficial in alcohol consumption. The variability in these studies may be due to different sample sizes, addiction to multiple substances of abuse in the subjects and compliance to the medication. Naltrexone shows some hepatotoxicity and makes the individual insensitive to safe doses of opiate analgesics for 72 hours [108-110].

2.13.5 Tiapride

Tiapride is a dopamine D2 antagonist, which is thought to help in the treatment of interminable alcohol addiction by decreasing the withdrawal side effects. Dopamine antagonists act by restraining the alcohol compensating impacts delivered by mesolimbic dopaminergic action and thereby forestalling strengthening impacts of alcohol. Dopamine agonists, for example, bromocriptine were tried in alcohol relapse as it was recommended that agonists can turn around the dopaminergic hypofunction caused in alcohol withdrawal, the clinical investigations, did not demonstrate viability contrasted with placebo [111].

2.13.6 SSRIs

SSRIs appear to have impacts in anticipating relapse in heavy drinkers who likewise experience the ill effects of depression. Buspirone, a 5 hydroxytryptamine (5HT)- 1A fractional agonist diminished alcohol utilization in animal studies about however was observed to be ineffectual in clinical examinations. Ondansetron, a 5HT-3 antagonist is observed to be viable in heavy drinkers, particularly those with serotonergic dysfunction. It acts by restraining alcohol actuated dopamine discharge in the mesolimbic framework [112,113].

2.13.7 Disulfiram and calcium carbimide

Disulfiram and calcium carbimide are aversive medications that act by restraining aldehyde dehydrogenase, which prompts accumulation of acetaldehyde, a metabolite of alcohol. This causes a few unsavory physiological manifestations, for example, tachycardia, flushing, diaphoresis, dyspnea, sickness, and heaving. The people shun alcohol consumption so as to evade these manifestations. In any case, clinical examinations have shown that there is no huge impact on these medications when contrasted with placebo [114].

2.13.8 Herbal medicines to treat alcoholism and withdrawal

For a few centuries, numerous therapeutic herbs are utilized for the ethanol reliance. Despite the fact that agents like benzodiazepines are the pillar for the executives of ethanol withdrawal, their utilization is related to symptoms, and hence endeavors are made to create herbal arrangements. Numerous herbal plants like *Pueraria lobata, Hypericum perforatum, Tabernanthe iboga, Salvia miltiorrhiza, Panax ginseng, Silybum marianum, Valeriana officinalis, Opuntia ficusindica, Scutellaria laterifolia, Oenothera biennis, Rosmarinus officinalis* etc. have been utilized for the treatment of ethanol abstinence and reliance [115,116].

2.13.9 Polyherbal remedies

Various plants *Ocimum sanctum, Withania somnifera,* and *Camellia sinesis* have been utilized as components of polyherbal preparations in the Ayurvedic arrangement of a drug for curing anxiety like manifestations (Table 2.1). Practitioners of Ayurvedic framework favor polyherbal preparations over individual agents as the Ayurvedic bargains expresses that a mix of medications guarantees the potentiation of the therapeutic adequacy of the main medication [117].

Table 2.1: Polyherbal remedies available in the market for alcohol withdrawal induced anxiety and depression [117].

Formulation	Mechanism	Uses
NR-ANXC (*Withania somnifera, Camellia sinesis, Ocimum sanctum,* Shilajit)	*Withania somnifera*–GABA mimetic activity, sedative property. *Camellia sinesis* –stimulating effect on the brain *Ocimum sanctum,* Shilajit decreases levels of anxiogenic mediators	Ethanol withdrawal-induced anxiety and depression

2.14 Animal models of alcohol withdrawal

Essential model of alcohol consumption and withdrawal in the rodents is voluntary alcohol consumption in which there is free choice between water and alcohol [118]. For a less

controlled methodology, alcohol can be furnished with the eating regimen, most usually as a component of a healthfully adjusted liquid diet that establishes the animal's sole source of sustenance and fluid [119]. Under these conditions, animals regularly expend adequate amounts of alcohol to achieve reliance as confirmed by the development of withdrawal symptoms at the point when the alcohol is discarded from the eating regimen. With this approach, the investigator controls the term of exposure, however, the animal decides the portion and pattern of alcohol utilization. The other methodology depends on voluntary alcohol utilization. These models more often than involve the utilization of animals that are hereditarily inclined to high alcohol inclination and drinking behavior; when given a choice among alcohol and water. Since numerous animals don't deliberately expend vast and reliable alcohol amounts, alcohol administration frequently is constrained that is, forced and controlled by the investigator. For instance, the alcohol might be delivered straightforwardly to the stomach by intragastric infusion, or the animal might be consistently presented to alcohol vapor in an inhalation chamber. Both of these techniques furnish the experimenter with a lot of deal over basic factors of alcohol presentation, for example, dose and timing. With these techniques, investigators can accomplish continued alcohol introduction (i.e., steady alcohol levels in the blood and brain) that outcomes in the advancement of withdrawal signs and manifestations once alcohol administration is ended [118-120].

2.15 *Withania somnifera*

2.15.1 Description

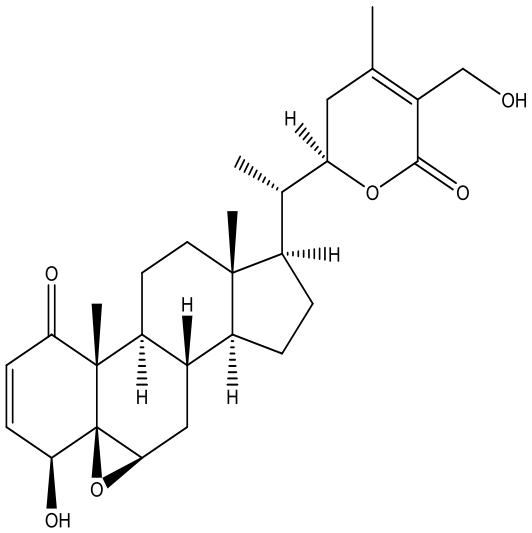
Withania somnifera (Ashwagandha) family Solanaceae is an essential old plant; their roots are utilized in Indian conventional system of medicine, Ayurveda and Unani (Fig. 2.5). As per Ayurveda, Ashwagandha is viewed as a standout amongst the most imperative herbs and the best adaptogenic. It contains the different important constituents like withanolides, cuseohygrine, anahygrine, tropine, anaferine, and glycosides, with starches and amino acids and so forth, Ashwagandha is additionally advantageous for the patient with anxiety [26,121].

Figure 2.5: *Withania somnifera* herb.

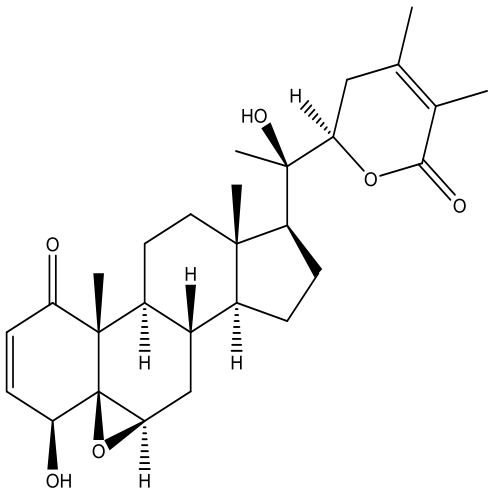
In India Ashwagandha grows in Haryana, Rajasthan, Punjab, Gujarat, Uttar Pradesh, Madhya Pradesh and Maharashtra. Ashwagandha is an evergreen expanding shrub having 30-150 cm height with glabrous and ovate leaves that are about 10 cm in length. Blooms are green or startling yellow, little around 1 cm long. Fruits are 6 mm in diameter, orange-red in color with yellow seeds [122].

2.15.2 Phytochemical studies

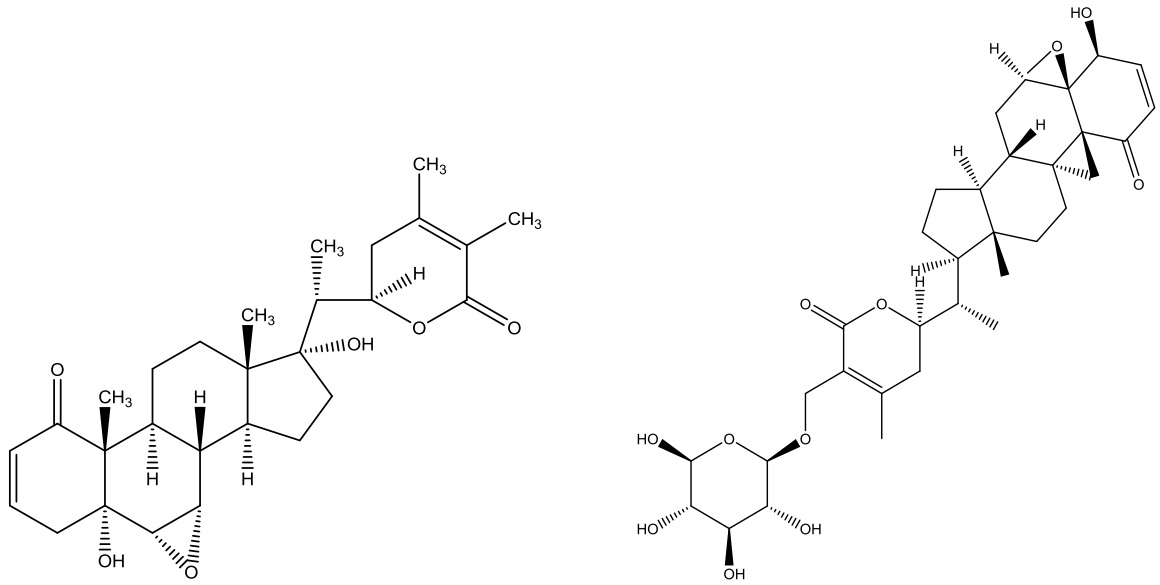
The biologically dynamic constituents present in *Withania somnifera* are alkaloids (cuscohygrine, anahygrine, tropine, ashwagandhine, and so forth), steroidal mixes, including steroidal lactones, withasomniferin-A, withasomidienone, withaferin A, withasomniferols A-C withanolides A-Y, withanone, sitoindoside VII to X and so forth [123-125]. The reported alkaloids in *Withania somnifera* are tropine, anaferine, isopelletierine, somniferine, cuscohygrine, dlisopelletierine, anahygrine, mesoanaferine, pseudotropine, choline, hygrine, ashwagandhine visamine, withanone, pseudowithanine, withananine and hentriacontane. Aside from these compounds *Withania somnifera* likewise contains phytoconstituents like glutamic acid, acylsteryl glucosides, withaniol, starch, reducing sugar, tyrosine, glycine, alanine, cysteine and tryptophan etc. [26,126].



Withaferin A



Withanolide D



Withanone **Sitoindoside IX**

Figure 2.6 : Structure of some chemical constituents present in *Withania somnifera* [127].

2.15.3 Pharmacology

Ashwagandha is as an important adaptogenic herb which has wide ranging health benefits and enhances the recovery of body's stress Ashwagandha is viable for sleep deprivation yet does not go about as a sedative. It is rejuvenating and nervine properties produce vitality which thus helps the body to settle and rest. Consequently, it causes the body to address a stress-related condition as opposed to veiling it with tranquilizers. Ashwagandha restores the sensory system, eradicates a sleeping disorder and facilitates stress. In joint inflammation, which includes joints that are painful, dry, swollen an aroused, Ashwagandha would be the herb of decision [26,127]. The drug comprises of the dried underlying roots of *Withania somnifera* which is authentic as a sedative in the pharmacopeia of India and its pharmacological action of the roots is credited to the presence of a few alkaloids. The root extract (70% alcoholic) has similar properties like alkaloids. *Withania somnifera* possess neuropharmacological, anti-inflammatory, antioxidant, analgesic, cardioprotective, immunomodulatory, antitumor, adaptogenic and antistress pharmacological properties [26]. Ashwagandha has been portrayed as a nervine tonic in Ayurveda. Ashwagandha is well utilized to advance brain and cognitive functions and may be utilized to treat Alzheimer's, Huntington's, Parkinson's and other neurodegenerative ailments. [127]. Various investigations have shown the GABA-mimetic action of *Withania somnifera* root extract. Phytoconstituents of Ashwagandha are reported to advance the development of nerves [128]. Ashwagandha

prompted a quieting anxiolytic impact that was similar to the medication Lorazepam. Ashwagandha likewise showed an antidepressant impact, practically identical with that actuated by imipramine. The examinations bolster the utilization of Ashwagandha as a state of mind stabilizer in clinical states of anxiety and depression [26,127].

2.16 *Centella asiatica*

2.16.1 Description

Centella asiatica family Umbelliferae (Apiceae), achieves height up to 15cm and growing to an elevation of 1800 m (Fig. 2.7). The plant grows mostly in muddy areas [129]. The plant is native to India, China, Sri-Lanka, Venezuela, South Africa, Mexico, South East USA and Columbia [130]. The stem is depilated, dappled and rooted at the nodes. *Centella asiatica* thrives broadly in obscure, mucky, clammy and moist places. Leaves are 1-3 from stem hub, long petiole, 2-5cm wide and 2-6cm long, orbicular-reniform with crenate edges.

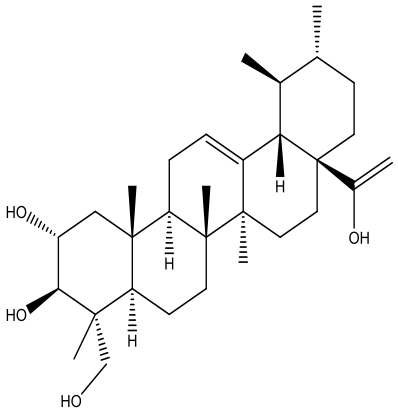
Figure 2.7: *Centella asiatica* herb.

Fruits are in knotted umbels, every umbel has 3-4 purple to white or pink blooms, blossoming generally happens in April to June. Fruits are approx. 2 inches in length, oval, spherical fit as a fiddle and firmly thickened pericarp. Seeds have a pedulous embryo which are along the side compacted [25,129].

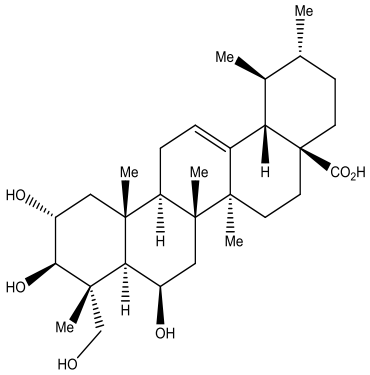
2.16.2 Phytochemical studies

The chief chemical constituents reported in *Centella asiatica* are Quercetin, kaempferol, caryophyllene, germacrene D, elemene, trans-farnesene, sitosterol, stigmasterol, asiatic acid, madecassic acid, madasiatic acid, asiaticoside, braminoside, brahmoside, brahminoside, thankuniside, isothankuniside, p-Cymol, pinene, terpene acetate and germacrene caryophyllene. Other constituents include urosolic acid, rosmarinic acid, resins, mucilages, and free amino acids [25,131]. The essential constituents of *Centella asiatica* are saponins,

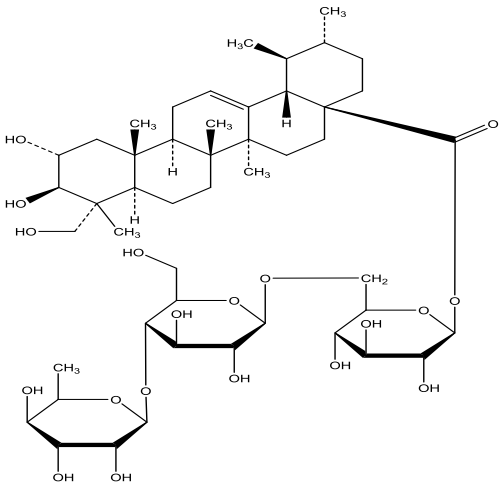
which includes asiaticoside, madasiatic acid and madecassoside. These triterpene saponins and their sapogenins are primarily in charge of the wound mending and vascular impacts by hindering the creation of collagen at the injury site [132,133].



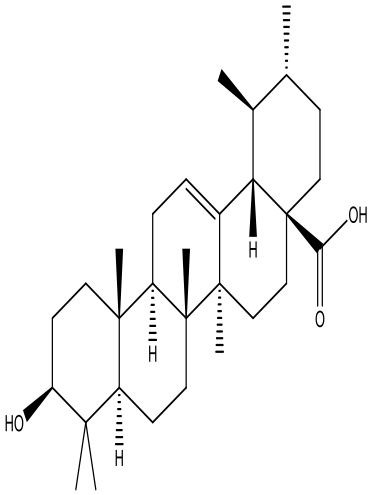
Asiatic Acid



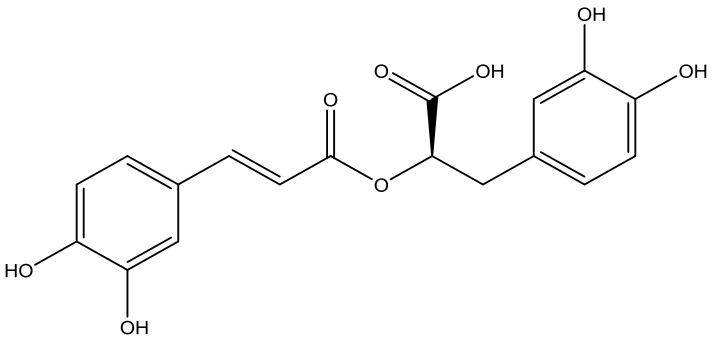
Madecassic acid



Asiaticoside



Urosolic Acid



Rosmarinic Acid

Figure 2.8: Structure of some chemical constituents present in *Centella asiatica* [134].

2.16.3 Pharmacology

Researchers have detailed distinctive pharmacological activities of *Centella asiatica* like wound healing, cytotoxic and antitumor, memory enhancing, cardioprotective, radio protective, antidepressant, slimming, immunomodulating, antiprotozoal, antitubercular and antileprotic [135-137]. The chief constituents like Brahmic acid, Brahmoside and Brahminoside are reported to have sedative, psychotropic and anticonvulsant activities. The plant is additionally helpful in anxiety, insanity and mental clutters like conditions [131]. In this way, Mentat a Polyherbal formulation containing *Centella asiatica* extract where every one of the plants components in a synergistic way delivers an enhancement of memory, concentration and consideration. The triterpenes in the plant are reported to have an antidepressant action [134]. Pectin separated from *Centella asiatica* indicated immunostimulating action and triterpenoid saponins and methanol extract demonstrated immunomodulatory impact [138].

2.17 *Camellia Sinensis*

2.17.1 Description

Camellia sinensis family Theaceae is commonly known as green tea (Fig. 2.9). It is 'non-fermented' tea and it consist a large number of catechins which are strong antioxidants. Tea drink is a mixture of the dried leaves of *Camellia sinesis*. It is a generally utilized therapeutic plant by the preliminaries all through India, China and prominent in the different indigenous arrangement of medication like Ayurveda, Unani, and Homeopathy [139,140]. By and by, it is cultivated in somewhere around 30 nations around the globe. Green tea has been expended all through the ages in India, China, Japan, and Thailand. *Camellia sinesis* is a bush that is normally cut to underneath six feet when grown for its leaves [28].

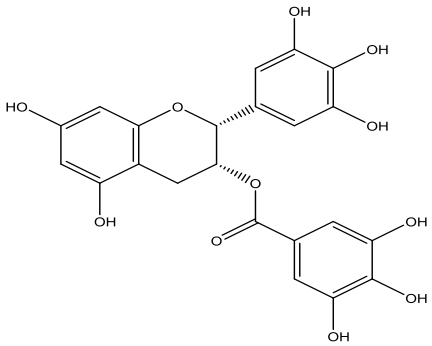
Figure 2.9: *Camellia sinensis* herb

The blossoms are whitish yellow, 2-4 cm in measurement having 6 to 8 petals. The seeds are squeezed to yield sweetish flavoring oil which is utilized for various therapeutic purposes.

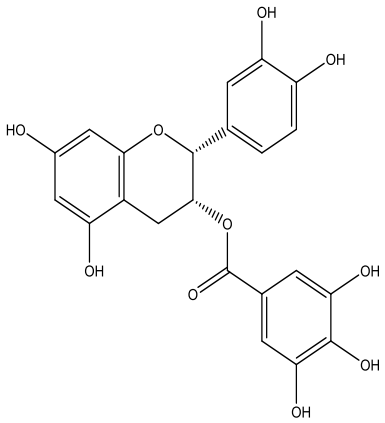
The leaves are 3.5-15 cm in length and 2.5-5 cm in width. The youthful, green leaves are ideally reaped for tea creation [28,139]. For the most part, initially a few leaves and the bud are harvested for processing. This hand picking is generally used for better quality as machine leads to damage [140].

2.17.2 Phytochemical studies

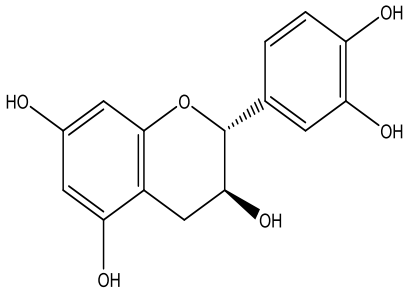
Camellia sinesis contain almost 4000 phytoconstituents out of which 33% are polyphenols [141]. The main constituents of this herbal plant, which are generally utilized for the medical reason, are alkaloids, sugars, amino acids, chlorophyll, proteins, volatile organic compounds, phenolic acids, aluminum, fluoride, trace components and minerals [142]. Most of the polyphenols found in *Camellia sinesis* are generally flavonoids. Catechins are believed to be in charge of the therapeutic benefits which have customarily been credited to green tea. Reported catechins in *Camellia sinesis* are epigallocatechin, epigallocatechin gallate, epicatechin and epicatechin gallate. The most effective catechin in green tea is epigallocatechin-3-gallate [139,143].



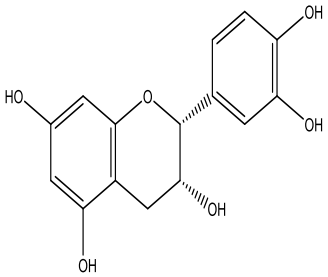
Epigallocatechin -3-gallate



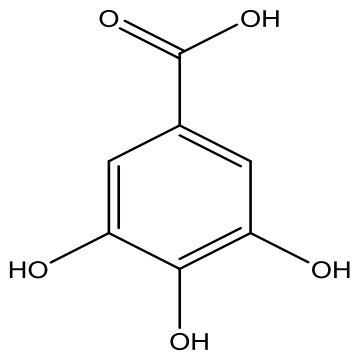
Epicatechin-3-O-gallate



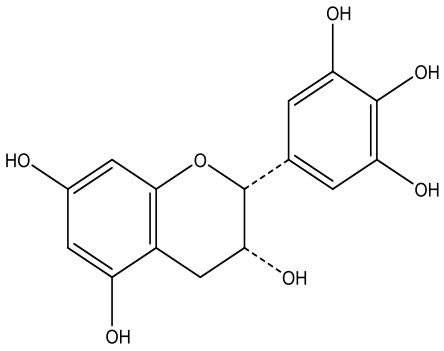
Catechin



Epicatechin



Gallic Acid



Epigallocatechin

Figure 2.10: Structure of some chemical constituents present in *Camellia sinesis* [139].

2.17.3 Pharmacology

Camellia sinensis have a considerable pharmacological activity and is broadly utilized for the treatment of many disorders. *Camellia sinensis* extract is reported to have anti-carcinogenic effects. The anticancer action of di-and tri-terpenes and other polyphenols present in *Camellia sinensis* is well reported [144]. Green tea has a protected effect against alcohol-induced oxidative stress in rodents [145,146]. A few examinations in animal models recommend that EGCG from *Camellia sinensis* may influence a few potential targets related to Alzheimer's [147,148]. Diminished hazard from cardiovascular infection and particularly from stroke was observed to be related to the expanded utilization of green tea, especially in ladies [149]. One investigation found a converse connection between the propensities for drinking more green tea day by day with having a background marked by stroke. An ongoing meta investigation of current literature discovered individuals devouring at least some green or black tea had 21% less shot of enduring a stroke [150]. Green tea has a rumored job in malignant growth counteractive action as tea catechins have been appeared to hinder tumor cell expansion just as advance the devastation of leukemia cells. Green tea and black tea polyphenols restrain cell development and actuate apoptosis of human cervical malignant growth cells [151,152]. Green tea is also reported to have an anti-diabetic impact [153]. Tea leaves are wealthy in fluoride, which is known to upgrade dental wellbeing and forestalls dental caries [154]. Green tea extracts standardized to 24.7% catechins and 8.35% caffeine is reported to invigorate brown fat tissue [155].

2.18 *Hypericum perforatum*

2.18.1 Description

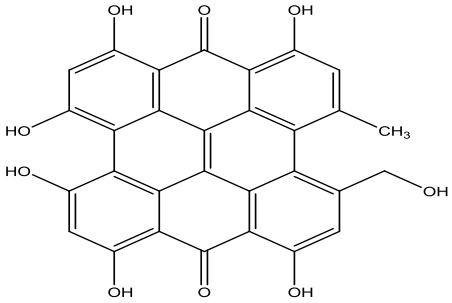
The Genus Hypericum containing 465 species all around is a substantial group of plants with potential restorative value. *Hypericum perforatum* is ordinarily known as St. John's wort (Fig. 2.11). *Hypericum perforatum* is indigenous to Northern Africa, Europe and Western Asia [23]. *Hypericum perforatum* comprises of bushes and herbs having yellow blooms with 4-5 petals with free branching normally range from 38 to 82 cm in height [156]. The leaves are smooth-margined that extend from 0.3-1.0 cm in width and 1.5 to 3 cm in length. The blossoms are five-petaled and yellow in color having 1.0-2.0 cm width. Smashed blooms produce a dark red shade. By pre-fall, the blooms produce capsules containing dim dark colored seeds [23,157]. The plant flourishes in poor soil usually in fields, knolls and squander territories. *Hypericum perforatum* is utilized since ancient Greeks as a natural solution for treating an assortment of inside and outside sicknesses. From ancient times plant has been utilized for treating anxiety, wound healing and infections. In most recent few decades, most of the research on *Hypericum perforatum* depended on its utilization as an antidepressant. Ongoing exploration proposes the viability of this herb in treating different illnesses including malignant growth, inflammation, and bacterial and viral sicknesses, and utilized as a cell reinforcement and neuroprotective agent [158,159].

Figure 2.11: *Hypericum perforatum* herb

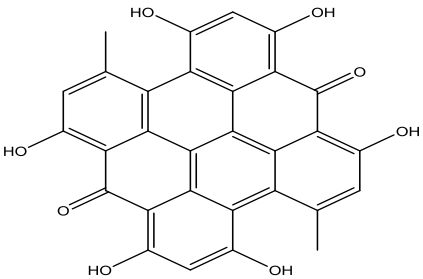
2.18.2 Phytochemical studies

Hypericum perforatum contains many biologically active compounds which include flavonoids, tannins, essential oil, phloroglucinols, procyanidins, xanthones, amino acids, phenylpropanes and naphthodianthrones etc. [160]. The most explored class of compounds secluded from *Hypericum perforatum* is naphthodianthrones which incorporate hypericin, pseudohypericin, protohypericin and isophypericin. Hypericin is found in the blooms as dark spots situated with the petals [161,162]. Flavonoids in *Hypericum perforatum* incorporate quercetin, kaempferol, luteolin, hyperside, rutin, biapigenin, hyperin, and myricetin. *Hypericum perforatum* contains a few classes of lipophilic compounds like oils and

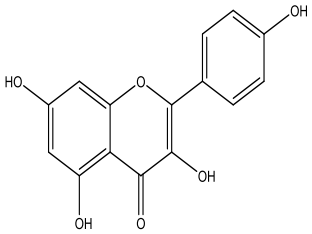
phloroglucinol subordinates [163,164]. Different phloroglucinols incorporate adhyperforin and furohyperforin. Fundamental oils comprise basically of mono and sesquiterpenes, α and β pinene, α terpineol and geranil. Other compounds incorporate tannins, hyperfolin, Xanthones, phenolic, hydrocarbons, carotenoids, gelatin and long chain alcohols [165].



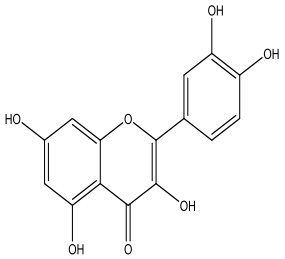
Pseudohypericin



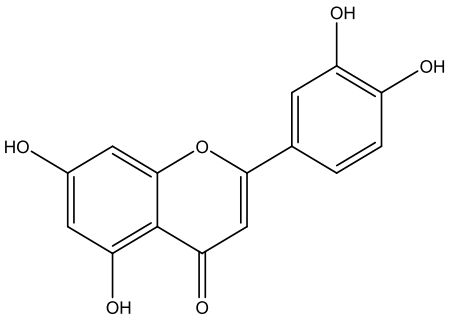
Isohypericin



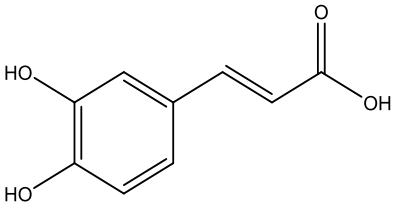
Kaempferol



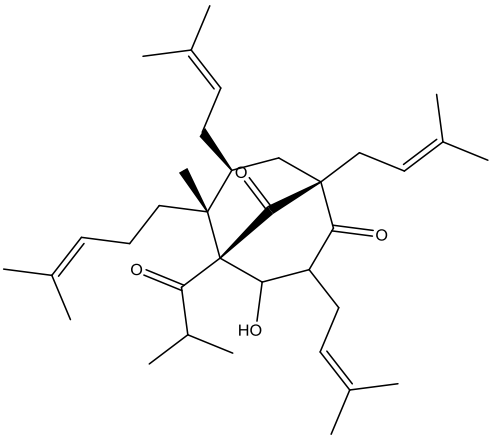
Quercetin



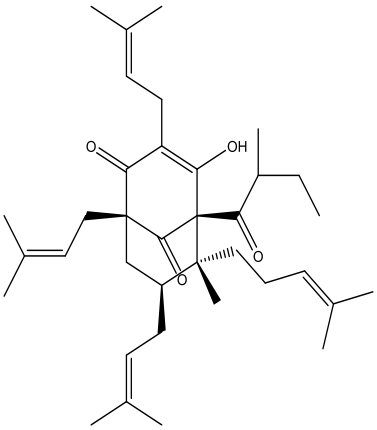
Luteolin



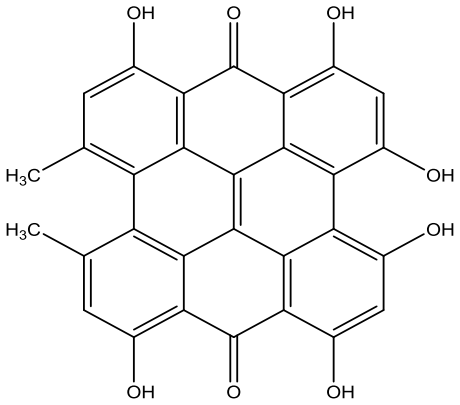
Caffeic acid



Hyperforin



Adhyperforin



Hypericin

Figure 2.12: Structure of some chemical constituents present in *Hypericum perforatum* [166].

2.18.3 Pharmacology

Numerous pharmacological activities, including antidepressant, anticancer, antibacterial and neuroprotective impacts have been archived for the extract of *Hypericum perforatum* or its chemical constituents. *Hypericum perforatum* is well known for its antidepressant properties. Several phytoconstituents of the plant including cyclopseudohypericin, isohypericin, hypericin, hyperforin, pseudohypericin, protohypericin, and a few different flavonoids are reported to have varying dimensions of contribution to its antidepressant activity [161]. One of the proposed mechanism of *Hypericum perforatum* as an antidepressant is the hindrance of the take-up of norepinephrine, dopamine and 5HT from the synaptic cleft of communicating axons [166]. Another contributing mechanism is the capability to tie to the GABA receptors which hinder the binding of GABA such decrease in GABA ligand binding results in

diminished depression [167]. Hyperforin is accounted for to have antibacterial activity against *Staphylococcus aureus*, *Streptococcus pyogenes* and *Corynebacterium diphtheriae*. Flavonoid and Catechin containing fractions of *Hypericum perforatum* have displayed activity against influenza infection. *Hypericum perforatum* is also reported for anticancer properties [158]. *Hypericum perforatum* fills in as a neuroprotective agent and reported for its beneficial effects as antiparkinsonian [156]. The extract of *Hypericum perforatum* decline oxidative stress and averts neurotoxicity, aggravation, and gastrointestinal issues and demonstrates guarantee as an anti-inflammatory agent [166].

2.19 *Bacopa monnieri*

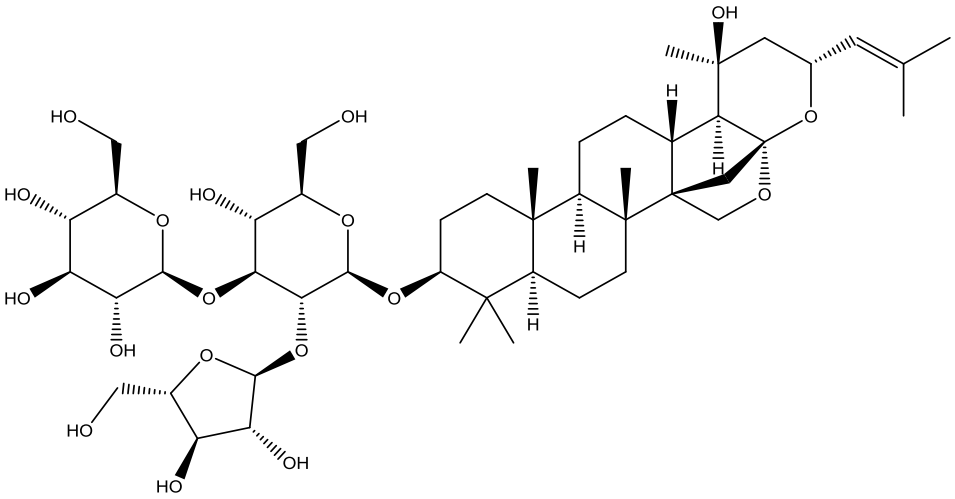
2.19.1 Description

Bacopa monnieri (family *Scrophulariaceae*) commonly known as Brahmi in India (Fig. 2.13). *Bacopa monnieri* is a dispersing, evergreen, plump herb [24]. *Bacopa monnieri* is a small quailing herb with various branches, little elongated leaves which are light purple in color initially and white when blossoms having 4 to 5 petals. Its branches increase on damp ground and form thick cushion. Roots are discovered developing at nodes. The leaves are little, the club formed, stalk less, and meaty which is harsh in taste [168]. The long stalk blooms are discovered single at the pivot of the leaves. Blooms are axillary, single, arranged on long slim pedicels. Seeds are minute and numerous while fruits are ovoid and tipped with style base. *Bacopa monnieri* generally found in clammy, boggy and moisten regions [169]. The plant is appropriated all through Sri Lanka, Nepal, India, China, Vietnam, southern states of the USA, Taiwan and Florida. The whole plant is utilized therapeutically**.** It was generally utilized in conventional medicine to treat different whines like epilepsy, insanity and asthma [170]. *Bacopa monnieri* is used broadly as a nootropic and to improve cognitive functions [168].

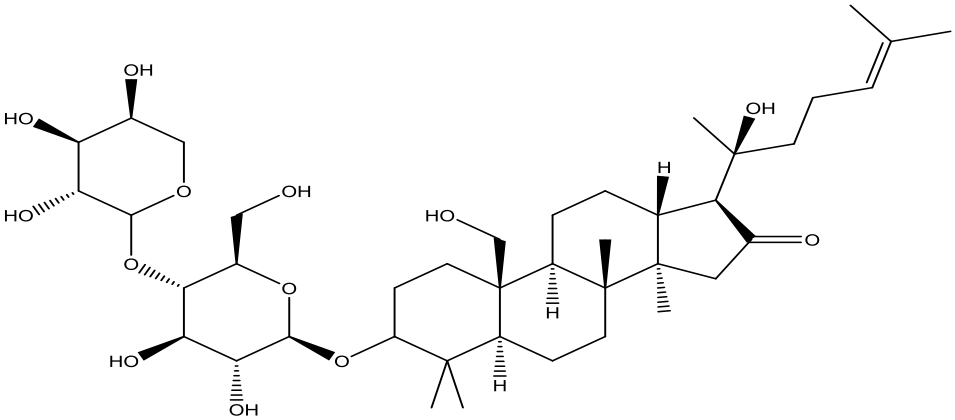
Figure 2.13: *Bacopa monnieri* herb

2.19.2 Phytochemical studies

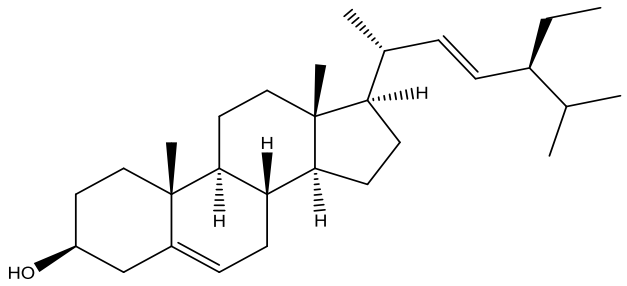
Bacopa monnieri contains compounds like alkaloids, sterols, and saponins. Different alkaloids like brahmine, herpestine and nicotine are reported in the plant [171]. Presence of a saponin, hersaponin, D-mannitol, potassium, β-sitosterol and stigmasterol is also reported in the plant. The phytoconstituent appear to be in charge of neuroprotective, nootropic and antiamnestic activity of the plant is bacoside A. Bacosides reported in the plant are bacogenin A1-4, Bacoside A1 and Bacoside A3 [171,172]. Another chemical constituent reported in the plant are bacopasaponins, bacopaside I and II (pseudojujubogenin glycosides), bacopasides III, IV and V(saponins), viz. monnierasides I– III and jujubogenin, named bacopasaponin G etc. [173-176].



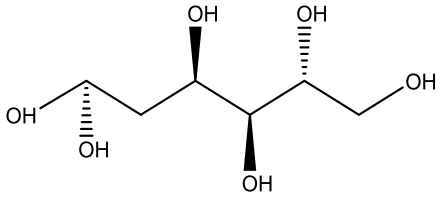
Bacoside A



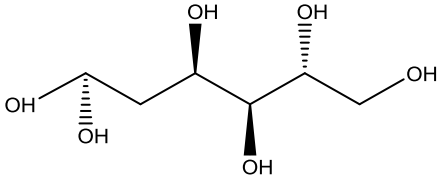
Bacoside B



Stigmasterol



β-Sitosterol



D mannitol

Figure 2.14: Structure of some chemical constituents present in *Bacopa monnieri* [177].

2.19.3 Pharmacology

Behavioral investigations in animals have demonstrated that *Bacopa monnieri* improves acquisition and retention, motor learning and delay annihilation of recently acquired behavior. Bacosides are reported to encourages temporal memory and weaken amnesia [178,179]. Critical psychological improving advantages have been exhibited with ceaseless administration of Bacopa extracts. Bacopa seemed to have different modes of action in the brain (i.e. pro-cholinergic, antioxidant, anti-inflammatory, improved blood flow, metal chelation, the expulsion of b-amyloid deposits, adaptogenic action) which may all be valuable in enhancing intellectual decline in the elderly [180]. The extract of *Bacopa monniera*, bacopasides I and II, bacosides A and B and bacopasaponin C showed antidepressant activity when studied on antidepressant models in rodents [181]. *Bacopa monnieri* extract has likewise reported to have antianxiety, adaptogenic, anticonvulsive and antioxidant activities. Plant additionally proved to normalize noradrenalin, Dopamine and 5-HT, in hippocampus of rodents in perpetual eccentric stress [182]. *Bacopa monniera*, is reported to have antiparkinsonian impacts by diminishing alpha-synuclein aggregation. Alcoholic extract of *Bacopa monnieri* showed a hepatoprotective and antiepileptic impacts [183,184]. In light of

numerous reports indicating vital pharmacological activities of *Bacopa monnieri*, the wide assortment of neuropharmacological activities of the plant opens up fascinating platform for future research and offers new points of view for curing the brain disorders [24,185].

2.20 *Ocimum sanctum*

2.20.1 Description

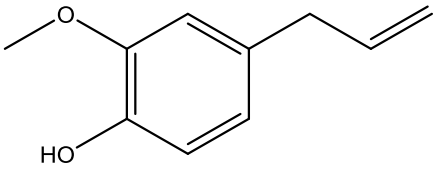
Ocimum sanctum family Labiatae is a branched, heavy and erect herb having height about 75 cm [27]. Plant is generally known as Tulsi and India's Holy Basil (Fig. 2.15). *Ocimum sanctum* is well utilized in Ayurveda for its therapeutic properties [186]. *Ocimum sanctum* is valuable in treating various manifestations like cold, hack, jungle fever, asthma, flu, heart issue, dengue, eye sicknesses, mouth diseases, creepy crawly nibbles, stress, bronchitis and kidney stones and so on [187]. *Ocimum sanctum* leaves are simple, sweet-smelling, fanned, inverse and curved having 5cm length. Blooms are lengthened and purple in shading and seeds are yellow and reddish. It is planted after blustery season and collected following a couple of months. Tulsi generally develops in warm and tropical areas up to 1800m above sea level. *Ocimum sanctum* is originated in India and broadly developed in certain territories of Africa and Asia [188-190].

Figure 2.15: *Ocimum sanctum* herb.

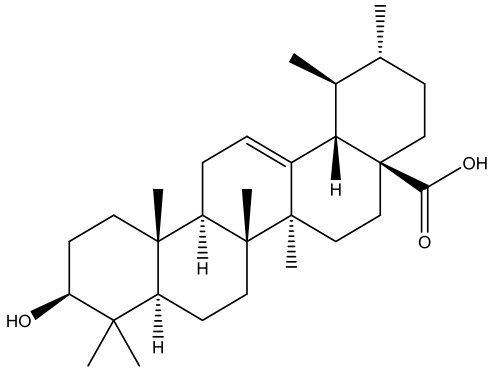
2.20.2 Phytochemical studies

The chemical composition of *Ocimum sanctum* is exceptionally complex, containing numerous supplements and other biologically dynamic compounds. One examination discovered forty-five compounds and oils in basil [27]. Its leaf contains volatile oil eugenol, euginal (additionally called eugenic acid), urosolic acid, limatrol, carvacrol, linalool, caryophyllene, methyl carvicol (likewise called Estragol), while the seed contains unsaturated

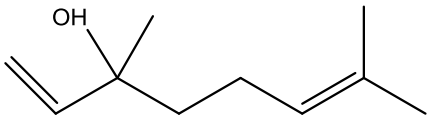
fats, sugars, sitosterol and the anthocyans [191]. The leaves and stem of *Ocimum sanctum* are reported to have phytoconstituents like saponins, flavonoids, triterpenoids, tannins, rosmarinic acid, isothymonin, cirsimaritin, isothymusin and apigenin [192]. The seeds contain an oil made out of unsaturated fats and sitosterol. It is one of the mending herbs that contain Vitamin A, and Vitamin C invigorates the creation of infection battling antibodies by up to 20% as well as cancer prevention agents that help to anticipate cell harm that can prompt destructive conditions [191,193].



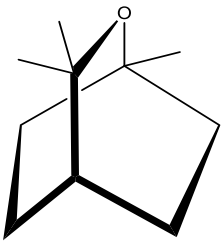
Eugenol



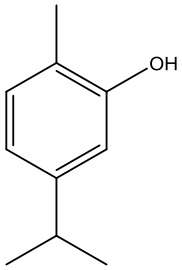
Urosolic acid



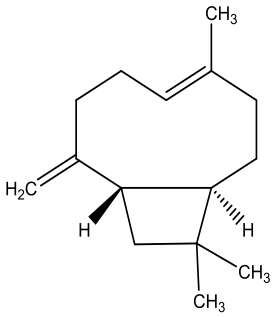
Linalool



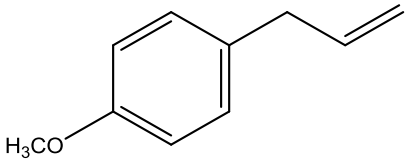
Cineole



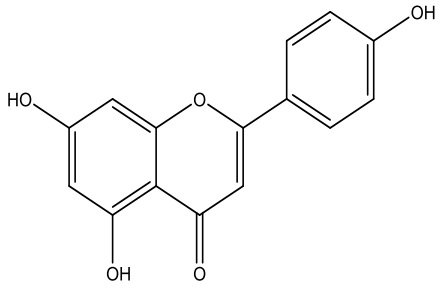
Carvacrol



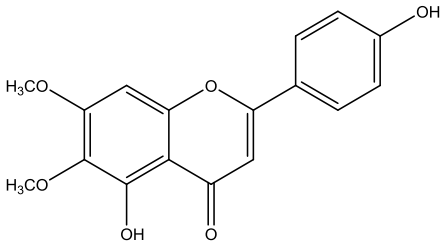
Caryophyllene



Estragole



Apigenin



Cirsimaritin

Figure 2.16: Structure of some chemical constituents present in *Oscimum sanctum* [191].

2.20.3 Pharmacology

Ocimum sanctum is utilized for several therapeutic properties like expectorant, pain relieving, antiemetic, diaphoretic, anticancer, antiasthmatic, antidiabetic, antifertility, hepatoprotective, hypotensive, hypolipidmic and antistress specialists [27,191]. *Ocimum sanctum* has likewise been utilized in the treatment of fever, bronchitis, joint inflammation, spasms and so on. In past examination, it has been demonstrated that the extract of *Ocimum sanctum* shows wide zones of inhibition against different bacteria [191,194]. Various examinations proposed that *Ocimum sanctum* diminishes the serum concentration of both cortisol and glucose and furthermore shown antiperoxidative impact. Subsequently, *Ocimum sanctum* may conceivably regulate corticosteroid-incited diabetic mellitus [195-197]. The ethanolic extract of *Ocimum sanctum* leaves has found to tweak carcinogen utilizing enzymes, for example, cytochrome-b5, cytochrome P-450 and aryl hydrocarbon hydroxylase of mice liver [191]. It was seen that ethanol and chloroform extract of *Ocimum sanctum* stem and leaf were successful in counteracting tonic seizures initiated by trans corneal electroshock [191,198].

Other investigation demonstrated that the plant has a critical impact on the central nervous system achieving the antistress and anxiolytic impact that may include the GABA-ergic framework. The tremendous study of the literature demonstrated that plant has a gigantic range of pharmacological properties [199].

2.21 Diazepam

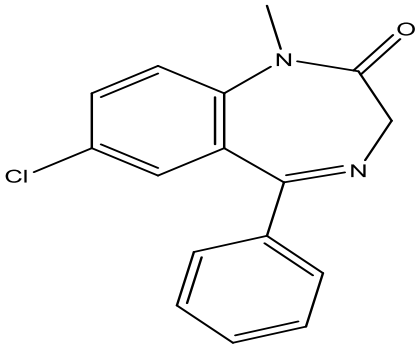


Fig.2.17: Diazepam

Diazepam is a derivative of benzodiazepine, a yellow crystalline compound having atomic weight of 284.75 and molecular formula is $C_{16}H_{13}ClN_2O$. Diazepam is insoluble in water and has antianxiety, sedative, anticonvulsant, muscle relaxant and amnesic impacts. Diazepam is reported to facilitate the activity of GABA. Diazepam accomplishes plasma concentration in 1 to 1.5 hours after oral consumption. Plasma protein binding of diazepam is 98%. Diazepam is metabolized to temazepam and N-desmethyldiazepam and both further processed to oxazepam. Diazepam and its metabolites are discharged for the most part in the urine, prevalently as their glucuronide conjugates [200,201].

2.22 Fluoxetine

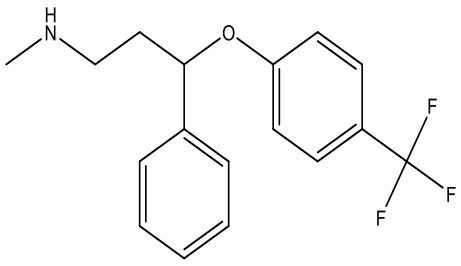


Fig.2.18: Fluoxetine

Fluoxetine is a U.S. Food and Drug Administration approved treatment for depressive disorders. Fluoxetine is a bicyclic subordinate of phenyl propylamine. Fluoxetine is the most

broadly utilized as SSRI and used for an assortment of neurological disorders including depression. Fluoxetine appears to encourage the serotonergic transmission in the focal sensory system by means of downregulation of presynaptic auto receptors. The oral bioavailability of fluoxetine is 72% which came to in 6 to 8 hours. 94% of the fluoxetine bounds to plasma proteins and have elimination half-life of 1-4 days. Fluoxetine is metabolized to norfluoxetine by means of *O*-demethylation [202,203].

2.23 Pentylenetetrazol (PTZ)

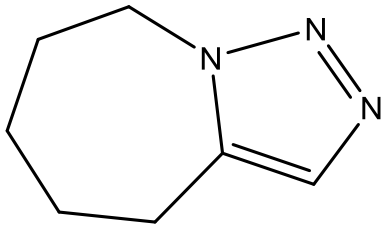


Fig 2.19: Pentylenetetrazol

PTZ is a tetrazol subordinate having convulsant activities in rodents apparently by disabling GABA-intervened inhibition through GABA receptor. PTZ is mostly utilized for the pharmacological screening of antiepileptic drugs. The studies of PTZ-instigated convulsions in rodents recommend its uses as a model of generalized and summed up tonic-clonic seizures. PTZ seizures are typically instigated by a solitary systemic administration [204,205].

CHAPTER 3

OBJECTIVES OF THE STUDY

- To prepare and standardize the POL-6 according to Indian Pharmacopoeia standards.
- To calculate the LD_{50} of the POL-6 according to OECD guidelines of acute and sub-acute toxicity in rats.
- To screen the POL-6 in ethanol abstinence-induced behaviors like anxiety, depression and seizures in rats and to carryout comparative mRNA expression studies in control and ethanol abstinence animals for $GABA_A$ receptor and NMDA-glutamate receptor subunits.

CHAPTER 4

MATERIALS AND METHODS

4.1 Materials

The solvents used in extraction like petroleum ether; ethanol were obtained from Loba Chemie, Mumbai India. 1, 1-diphenyl-2-picrylhydrazyl (DPPH), Naphthylethylenediamine dichloride (NEDA), Vanillin, Aluminium trichloride ($AlCl_3$) was purchased from Himedia Laboratories, Mumbai, India. Hydrogen peroxide was procured from Finar chemicals, Ahmedabad, India. Lead acetate was procured from Central drug house, Delhi, India. Standard markers used in HPTLC studies like Withaferin A, β-setosterol, Caffeic acid, Rutin, Catechin and Quercetin were procured from Oxford Laboratory, Mumbai, India. Trichloroacetic acids (TCA), Ehrlich's reagent, Folin-Ciocalteu's reagent, Ferric chloride were procured from Loba chem., Mumbai, India. Formaldehyde solution was procured from CDH, New Delhi, India. The biochemical kits were procured from Erba diagnostics Mannheim, Solan, India and Loba chem., Mumbai India. Verso cDNA synthesis kit was procured from Thermo Scientific, India. SYBR green super mix for RT-PCR was procured from BIO-RAD, India. Analytical grade chemicals and reagents were used in present study.

4.2 Equipment and instruments

UV (Ultra violet) double beam spectrophotometer from Thermo scientific, Water bath and Vortex shaker from REMI Mumbai, India, lyophilizer (freeze dryer) from New Brunswick, refrigerated centrifuge (Remi Electronik Ltd., Model no. CPR-24 plus); India; Sonicator (Ultrasonic cleaner; Model Cleaner 30A), Rota evaporator from Heidolph, Germany Pvt Ltd; deep freezer from Cell frost Innovation Pvt. Ltd. Model-CF 300, HPTLC having CAMAG Linomat 4 sample applicator (Camag Muttenz, Switzerland) equipped with syringe (Hamilton), twin trough chamber (CAMAG), TLC scanner 2 conjugated with winCATS software, Real-time PCR analyzer CFX96 from Bio-Rad, India were used.

4.3 Plant material

The collection of *Hypericum perforatum* L. aerial parts was done from the physic garden of Jaypee University of information technology, Himachal Pradesh (H.P.), India and validated by Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Solan, H. P. (Field book number: 13420). The dried leaves of *Camellia sinensis* (Batch No. ERM–23) and

Ocimum sanctum (Batch No. RHD 283), dried roots of *Withania somnifera* (Batch No. EBD–18) and dried entire plant of *Centella asiatica* (Batch No. ERD–040) and *Bacopa monnieri* (Batch No. ERD–92) were procured from Natural Remedies, Bangalore, India.

4.3.1 Plant material extraction

The fresh material from six plants was washed, shade dried cut into small pieces individually pulverized to form coarse powder (confirmed by passing through sieve number 60). The coarse powdered plant material placed in soxhlation assembly, defatted with pet. ether (30–40 °C). After defatting, plant material was air dried and then further exposed for 48 hrs at 50°C using hydro alcoholic extraction (70% v/v). The acquired solvent was then removed using rotatory evaporator. The semisolid mass obtained after rotatory evaporation was further lyophilized to obtain dry powder and was refrigerated at 2–8 °C individually for further use.

4.4 POL–6 preparation

POL-6 was prepared by utilizing the effective doses of all the six plants. The effective doses of the plants were selected from the literature. (Table 4.1)

Table 4.1: The most effective doses of the plants.

Sr. No.	Plant	Effective Dose	Major Symptoms Recovered
1.	*Bacopa monnieri*	200mg/kg	Depression [206]
2.	*Hypericum perforatum*	100mg/kg	Depression [207]
3.	*Centella asiatica*	200mg/kg	Seizures [208]
4.	*Withania somnifera*	200mg/kg	Anxiety [209]
5.	*Camellia sinensis*	100mg/kg	Anxiety [210]
6.	*Ocimum sanctum*	200mg/kg	Seizures [211]

The dried powdered extract of the plants (*Bacopa monnieri, Hypericum perforatum, Centella asiatica, Withania somnifera, Camellia sinesis and Ocimum sanctum*) was weighed

independently and blended in the ratio (2:1:2:2:1:2) respectively utilizing a twofold cone blender. To get a homogeneous mix blend was further sieved to mesh size 40 and kept in a firmly clean container (closed) away from heat, moisture until further use.

4.4.1 Organoleptic assessment of POL-6

Sensory characteristics of POL-6 like color, odor, shape, size, taste, texture were recognized utilizing organoleptic assessment [212].

4.4.2 Physicochemical investigation and solvent extractive values of Pol-6

4.4.2.1 Loss on drying

Loss on drying is a test method used widely to determine the moisture content or the volatile content loss from the sample. It is expressed as percent w/w. The POL-6(10g) drying was conducted in a flat weighing bottle at 105°C for 5 hrs. The calculation of percentage was done with reference weight initially [213].

4.4.2.2 Total ash and acid insoluble ash determination

In Indian Pharmacopoeia total ash value should be <10 % and elevated ash value is an indication of adulteration, contamination and carelessness in preparing the sample. In this test method, 2g of POL-6 was weighed accurately and placed in previously ignited and recently tared weighed crucible of silica. The powdered POL-6 was burned in a muffle furnace for 5-6 hours by gradual elevation in the temperature (not surpassing 450°C), till the appearance of white color which indicated sample as carbon free. After cooling in desiccator the material was weighed and the value of total ash was determined by calculating difference in empty crucible weight and crucible containing total ash. Total ash percentage was calculated further using total ash/weight of plant material x 100. For acid insoluble ash determination, 25ml of dilute hydrochloric acid was added to crucible having total ash, further boiled for 5 min having a covering of watch glass, After 5 minutes the hot water was used to rinse the watch glass and the insoluble matter obtained was collected on an filter paper (ashless) and ignited. In comparison to the air-dried drug the acid insoluble ash % was found [213].

4.4.2.3 Alcohol soluble extractive value

In a closed conical flask 100 ml of alcohol was added to 5g of air-dried POL-6 and kept for 24 hrs, along with shaking at regular interval and the mixture was kept 18 hours. After a period of 24 hours the extract was filtered by the help of filter paper. Filtrate (25ml) was

evaporated to dryness at 105°C and the semisolid content was weighed. The % of alcohol dissolvable extractive value was determined with POL 6 as reference [212].

4.4.2.4 Water soluble extractive value

In a closed conical flask 100 ml of water was added to 5g of air-dried POL-6 and kept for 24 hrs, along with shaking at regular interval and the mixture was kept 18 hours. After a period of 24 hours the dissolvable was filtered by the help of filter paper. Filtrate (25ml) was evaporated to dryness at 105°C and the semisolid content was weighed. The % of water dissolvable extractive value was determined with POL 6 as reference [212].

4.4.2.5 Determination of pH

For pH determination, 1% solution of POL-6 was prepared in distilled water. The pH of the solution was determined using pH meter [213].

4.4.3 Physical characteristics of POL-6

4.4.3.1 Bulk density and tap density

100g of POL-6 powder was weighed and added to measuring cylinder. The initial volume was noted and the value of bulk density was derived using formula:

Db = M/Vb, M = Powder mass and Vb = Packed powder volume

The tapped density was calculated by tapping powder in a measuring cylinder on flat table surface until no further decrease in volume was noted.

Tapped density = Powder weight / Powder volume after tapping [212]

4.4.3.2 Angle of repose

As per Indian Pharmacopoeia angle near 25° relate to excellent flow properties of powders and the powders having angle of repose more than 50° have unsuitable flow properties. In this method, a funnel is kept with its tip at a given height 2.5 cm (H), over the graph paper that is placed on horizontally flat surface. Pouring of the powder was done very carefully till they form a conical heap. The equation used for calculating angle of repose is $\tan \alpha = H/R$, where α is the angle of repose, R being the radius of the conical shaped heap [212].

4.4.3.3 Hausner’s ratio

Hausner's ratio predict the indication of flow properties of powder and related to the inter particle friction in the powders. The equation of measuring: Hausner’s ratio =Tapped density/Bulk density.

Coarse powders have low inter particle friction and generally have Hausner’s ratio of 1.2, while powders with Hausner’s ratio more than 1.6 are classified as more cohesive, less flowable [213].

4.4.3.4 Carr’s index

Carr's index is the characteristic of powder flowability. It measures the powder flow from the bulk density. Carr index value more than 25% shows poor flowability while 5% to 15% shows excellent flowability. The equation used for estimating is:

Carr's index = (Tapped density –Bulk density/ Bulk density) × 100 [213].

4.5 Qualitative phytochemical screening

The qualitative phytochemical analysis of the individual plant extracts and POL-6 was performed for determining the presence of major bioactive phytoconstituents like flavonoids, glycosides, alkaloids, phenolic, sugars, tannins, saponin, fixed oils, steroids and terpenoids etc. according to the method described by Kokatte, 1995 [214-216].

4.6 Quantitative screening of POL-6

4.6.1 Total flavonoid content quantification

Total flavonoid content in POL-6 was calculated by aluminum chloride assay method and rutin was used as a reference compound. A volume of 0.5 ml POL-6, distilled water (2ml) and 0.15 ml of sodium nitrite (5% w/v) was made. Add 0.15 ml of aluminum trichloride (10%) after 5 min, and after the incubation of 6 min, To a reaction mixture, 2ml of NaOH (4% w/v) was added. 5ml of total volume was made with distilled water and incubated for more 15 min. When the pink color is observed in the above mixture, absorbance was measured against blank at 510 nm. The total flavonoid content was represented in mg of rutin equivalent per gram of POL-6 [217].

4.6.2 Total phenolic content quantification

The determination of phenolic content in POL-6 was done using Folin and Ciocalteu method was used for calculating the. Folin and Ciocalteu phenol reagent was mixed with 100µl of

test in 1:10 proportion. The sample mixture was incubated for 1 min at room temperature followed by addition of 1.5 ml of 20% sodium carbonate. The reaction mixture (10 ml) was incubated at room temperature for 90 min (in dark). The sample absorbance was read at 725nm and phenolic content was calculated on the basis of Gallic acid equivalents, GAE/g of dry plant material [218].

4.6.3 Quantification of condensed tannin content

400µL of POL-6 was added to hydrochloric acid concentrated (1.5 ml) and 4% solution of vanillin (3 ml) in methanol. The mixture was incubated for 15 min and the absorbance was taken at 500 nm. The tannin content was expressed as mgCE/g of test, utilizing a Catechin calibration curve [219].

4.7 Qualitative phytochemical profiling of POL-6 by HPLC

POL-6 (5mg) was dissolved in 10mL methanol (80%) and 25ppm solution was made by diluting it further. The solution was filtered with 0.22 micron syringe filter. The HPLC system (Agilent Technologies) consists of an LC-binary pump, diode array detector, EZ-chrom system controller, and Innoval C18 (4.6×250mm) column. For separation, 0.14 g of anhydrous potassium dihydrogen orthophosphate (KH_2PO_4) was added to 900ml of HPLC grade water and orthophosphoric acid (0.5ml) was dissolved in above mixture. The volume of the mixture was made upto1000 ml with water and then filtered through membrane filter (0.45 µ). After filtration the solution was placed in a sonicator for 3 minutes. The prepared solution was considered as the mobile phase gradient solvent (A) and the Acetonitrile was used as the solvent (B). 20µl sample was injected through the SIL-HTC Shimadzu Autosampler. The conditions for solvent system were 20:80 ratios, flow rate 1.5ml/min and a run time of approximately 45 minutes. The chromatogram was obtained at wavelength of 227nm [220].

4.8 Liquid Chromatography- mass spectroscopy profiling of POL-6

The major chemical constituents present in the POL-6 were identified by using liquid chromatography-mass spectroscopy (LC-MS). Phenomenex C18 (150 × 4 mm i.d., 5 µ) with single quadrupole mass spectrometry analyzer was used for the liquid chromatography separation. 0.5% formic acid–acetonitrile (75:25%) was used as the mobile phase. The flow rate was 0.5 ml/min. The solvent was controlled by isocratic elution. The column temperature was kept at 30°C. The MS spectra were gained in the positive ion mode. Nebulizing pressure

of the drying gas (N_2) was of 25 psi, temperature was 350^{o}C at a gas flow rate of 6 ml/min. About 0.5 g of POL-6 was diluted with methanol and filtered with 0.22 µm nylon filter prior to examination. A 5 µl volume of the POL-6 was injected onto the column for examination. The mass fragmentations were identified by using spectrum database for organic compounds [221].

4.9 Quantification of major constituents present in POL-6 by HPTLC

The major constituents like Withaferin A, Quercetin, β-sitosterol, Catechin, Caffeic acid and Rutin were quantified in the POL-6 using a HPTLC instrument with CAMAG Linomat V automatic sample applicator, TLC scanner III, Camag twin trough chamber 10×10 cm, and WinCATS software in present study. POL-6 (suspended in methanol) and standard solutions (each 5 µl) (suspended in methanol) were applied in the form of a band having bandwidth 8 mm; distance between the bands 14 mm and a constant application rate of 150 nL s^{-1} using a microsyringe (Hamilton-Bonaduz Schweiz, Linomat syringe, 500 µl size) to a silica gel precoated 60 F254 TLC plates (10 × 10 cm with 200 µm thickness). TLC plates were then placed under the different mobile phases (Withaferin A; chloroform: methanol,:0.5, v/v, Quercetin; Toluene: methanol, 7:3, v/v, β-sitosterol; n-hexane: ethyl acetate, 8:2, v/v, Catechin, Caffeic acid; toluene: ethyl acetate: formic acid, 5:4:1, v/v/v, Rutin; ethyl acetate: formic acid: acetic acid: water, 10:1.1:1.1:2.6, v/v/v/v) in a glass developing chamber and development was performed in ascending manner to a distance of 8 cm. After the development, the densitometric scanning of the air-dried plate was performed by the help of TLC scanner operated in reflectance-absorbance mode, slit dimensions: 6×0.45 at 254 nm. The calibration curve of all the standards was drawn. The sample and standard spots were applied on TLC plates and the contents of metabolites were analyzed using regression equation from the calibration plot and expressed as % w/w [222].

4.10 Evaluation of antioxidant activity of POL-6

4.10.1 Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

POL-6 was assayed for the DPPH scavenging activity as per its ability to bleach the stable DPPH radicals. Different concentrations 5, 10, 20, 40, 80, 100µg/ml of POL-6 and standard (ascorbic acid) were prepared. 500µl of each concentration was mixed with 500µl of DPPH. In the control test tube, 500µl of DMSO and 500µl of DPPH was mixed which will serve as an absorbance control. The absorbance (Abs) was taken at 517nm after incubating all the

samples at 37^{o}C for 30 minutes in the dark. The equation (Scavenging DPPH (%) = [(control Abs –sample Abs)/control Abs] × 100) was used for calculating the percentage inhibition of the samples. Further IC_{50} was calculated from the percentage inhibition which shows the level at which 50% of the free radicals was scavenged by the test sample [223].

4.10.2 Nitric oxide scavenging assay

Nitric oxide when reacts with oxygen produces nitrate and nitrite; the quantities of these stable products can be determined by using Griess reagent method. At various concentrations (5, 10, 20, 40, 80 and 100 µg/ml) POL-6 and ascorbic acid standard were prepared. This was followed by incubation period at 37°C for 5 hrs with the addition of 5mM of sodium nitroprusside solution in 0.025 M phosphate buffer (pH-7.4). A proportionate measure of methanol was served as a control. After incubation 500µl of incubated solution was diluted with 500µl of Griess reagent and absorbance was measured at 546 nm. The percentage scavenging or antioxidant by test samples at different concentrations was analyzed using equation (Scavenging NO (%) = [(Abs control –Abs sample)/Abs control] × 100) further IC_{50} value was calculated [224].

4.11 Animals

Wistar rats of either sex were bought from the NIPER, Punjab, India and housed at Animal House, Jaypee University, Solan, H.P. They were acclimatized to laboratory conditions kept at temperature 23±2ºC, light and dark cycle (12:12 hrs). Animals were sustained with nutritional pellets diet (Aashirwad Industries, Chandigarh, India) and water *ad libitum*. The protocol was duly approved from IAEC, Jaypee University, Solan, H.P. India (3/GLG/2014/JUIT/IAEC). It was conducted in strict compliance with internationally accepted principles for laboratory animal use and care and as per the guidelines by Committee for the purpose of Control and Supervision of Experiments on Animals standards (1716/PO/a/13/CPCSEA).

4.12 Acute toxicity assessment

Acute toxicity of POL-6 was assessed in accordance to OECD-2008 test rules 425. Ten healthy non-pregnant and nulliparous female Wistar rats were randomized into two groups; negative control and another group was treatment control. Initially, POL-6 was administered orally at a single dose of 2000 mg/kg to one female rat from the treatment control group

(Table 4.2). Following 48 hrs the same dose was administered to the additional 4 female rats in the group. The same administration protocol was followed to in the negative control group (five female rats) administered with 1% sodium carboxymethyl cellulose. After POL-6 treatment all animals were closely observed for general action, muscle coordination, lacrimation, salivation, piloerection, tremors, straub, ptosis, corneal reflexes, sedation, seizures, anesthesia and mortality normally for 30 min and afterward intermittently for 4 and 24 hrs. Animals were observed for 14 days and water intake, body weight and feed utilization were recorded daily [225]. On the 14^{th} day of the study animals were sacrificed by cervical dislocation and subjected to the necropsies.

Table 4.2: Experimental design for acute toxicity study.

Sr. No.	Group	No. of Animals	Treatment
1.	Group 1 (Normal Control)	6 (Female)	Water + CMC
2.	Group 2 (POL-6 Treated)	6 (Female)	POL-6 (2000 mg/kg)

4.13 Sub-acute toxicity assessment

The sub-acute toxicity study was assessed in accordance to the OECD guidelines 407. 52 Wistar rats both male and female were used for the study. Animals were assigned randomly into five groups (Table 4.3).

Table 4.3: Experimental design for sub-acute toxicity study.

Sr. No.	Group	No. of Animals	Treatment
1.	Group 1 (Control)	6	Water + CMC
2.	Group 2 (Low Dose)	10	POL-6 (250 mg/kg)
3.	Group 3 (Intermediate Dose)	10	POL-6 (500 mg/kg)
4.	Group 4 (High Dose)	10	POL-6 (1000 mg/kg)
5.	Group 5 (Control Satellite)	6	Water + CMC
6.	Group 6 (High Dose Satellite)	10	Pol-6 (1000 mg/kg)

The Group 1(control group) consists of male and female rats; 6 each and received sodium CMC (1%) vehicle treatment daily at 1mL/100g body weight dosage for 28 days and on 29th day 6 rats (male and females; 3 each) were sacrificed and remaining 6 were taken as control satellite group (Group 6). Animals of Group 2, Group 3 and Group 4 consist of five male and five female rats. POL-6 at the doses of 250, 500 and 1000 mg/kg orally was administered daily to the animals of Group 2, 3, 4 for 28 days. Another group (Group 5) consisting of 5 male and 5 female rats was taken as a high dose satellite group (1000 mg/kg, oral administration) for 28 days; no treatment was given for succeeding 14 days. On 43rd day, animals of Group 5 were sacrificed and assessment of aggravation, persistence or the varying adverse effects was done. Change in body weight, feed utilization and water intake by the rats were noted during the experimental period [226].

4.13.1 Haemato–biochemical parameters

Blood samples were collected from retro-orbital sinus of the animals using EDTA coated tubes for hematological analysis (Table 4.4). For biochemical analysis blood was taken in the falcon tubes and centrifuged (3000 RPM) for 10 min at 4°C [228].

Table 4.4: Hematological parameters and Biochemical parameters in toxicity studies.

Hematological parameters	**Biochemical Parameters**
White blood cell count (WBC)	Serum glutamate-pyruvate transaminase (SGPT)
Monocytes (MON)	Serum glutamic-oxaloacetic transaminase (SGOT)
Lymphocytes (LYM)	Blood urea
Eosinophil (EOS)	Albumin (ALB)
Neutrophil (NEU)	Globulin
Total cholesterol (CHOL)	Alkaline phosphatase (ALP)
Platelet count (PLT)	Serum uric acid
Packed cell volume (PCV)	Serum creatinine
Red blood cell count (RBC)	Total serum protein
Hemoglobin count (HGB),	
Mean corpuscular volume (MCV)	
Mean corpuscular hemoglobin (HGB),	
Erythrocyte sedimentation rate (ESR)	

4.13.2 Organ weight and histopathology

At the termination of the study animals were sacrificed by euthanasia and gross necroscopy was performed in detail. Organ weights of spleen, brain, heart, kidneys, thymus, liver, epididymis/uterus, adrenals and testicles/ovaries were measured. The ratio of each organ to terminal body weight was calculated and relatively expressed as g/100 g of body weight. All the major organs were fixed in 10% formalin includes brain, liver, heart, kidneys, lungs, spleen, pancreas, ovaries. To check any cellular damage, 5 μm histological sections of the tissues were stained using hematoxylin and eosin (H&E) followed by microscopic examination [229,230].

4.14 Ethanol withdrawal study

Animals were divided randomly into 7 groups (n=6) and housed separately (Table 4.5). Group 1 received liquid diet; Group 2 was subjected to voluntarily ethanol intake for 15 days and received the vehicle 0.5% carboxymethyl cellulose (CMC) during ethanol withdrawal days (16^{th}, 17^{th} and 18^{th} day). Group 3, 4 and 5 were given alcohol treatment for 15 days and during ethanol withdrawal days received POL-6 (20, 50 and 100 mg/kg/, p.o.) once a day, respectively. Group 6 received alcohol treatment for 15 days and during ethanol withdrawal period diazepam (2mg/kg, p.o.) was administered once a day, Group 7 received alcohol treatment for 15 days and fluoxetine (5mg/kg, p.o.) was administered once a day during ethanol withdrawal period. Alcohol treatment was given to the animals as per described in the earlier studies [231]. The alcohol fed animals were allowed to have free intake of 4.5%v/v ethanol on the 1^{st} day, 7.5%v/v ethanol on the 2^{nd} day and 9% v/v ethanol from third day to 15^{th} day with a 2 bottle choice paradigm (water vs ethanol). On 16^{th} day alcohol was withdrawn and liquid diet (alcohol free) was introduced to the alcohol fed animals while control group animals were continued on the same diet. As per earlier studies the peak level of withdrawal symptoms were observed on 3^{rd} day of withdrawal i.e. on 18^{th} day. Hence animals in the present study were subjected to the behavior parameters analysis on day 18^{th} only. One hour after the last dose of the drug treatment animals were individually tested for examining the anxiety on the elevated plus maze and light-dark test. Depression parameters were studied by exposing the animals to the tail suspension test and forced swim test. Effect of alcohol withdrawal on locomotor activity was studied by exposing the animals to the actophotometer. To study the alcohol withdrawal seizures a sub convulsive dose of the Pentylenetetrazol (30mg/kg i.p.) was injected to the animals and seizure scoring was

recorded. After behavior studies blood was withdrawn immediately through retro orbital of rats for hematological examination and serum was separated for examination of biochemical parameters. Body weight change and ethanol intake of the animals were recorded every day during the study and expressed as g/kg/day. After completion of the study animals were sacrificed by cervical dislocation and brain was isolated. The amygdala, hippocampus and prefrontal cortex were isolated from the rat brain for further analysis of variations in mRNA expression by RT-PCR (Fig. 4.1) [232-234].

Table 4.5: Experimental design for alcohol withdrawal study.

S.N.	Group (n=6)	Treatment
1.	Group 1 (Normal Control)	Water + CMC
2.	Group 2 (Disease Control)	Water + ethanol + CMC
3.	Group 3 (POL-6 Treatment 1)	Ethanol + POL-6 (20 mg/kg)
4.	Group 4 (POL-6 Treatment 2)	Ethanol + POL-6 (50 mg/kg)
5.	Group 5 (POL-6 Treatment 3)	Ethanol + POL-6 (100 mg/kg)
6.	Group 6 (Standard 1)	Ethanol + Diazepam (2mg/kg)
7.	Standard (Standard 2)	Ethanol + Fluoxetine (5 mg/kg)

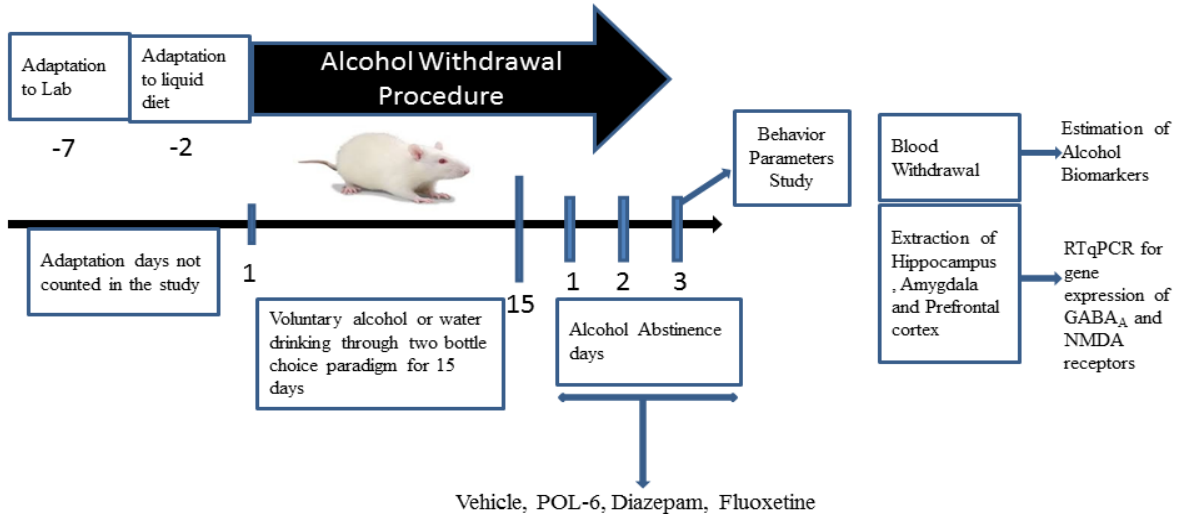


Fig 4.1 Experimental design for alcohol withdrawal study.

4.14.1 Blood alcohol concentration measurement

After consistent deliberate ethanol intake by the animals for 15 days blood was collected from the tail vein on day 15^{th} and on 16^{th}, 17^{th} and 18^{th} day (alcohol withdrawal period) in EDTA coated vials. Blood containing vials then centrifuged (1500×g) at 4 °C for 5-8 min. Plasma was stored at −20°C. BAC was determined by using bioassay systems' EnzyChrom ethanol assay kit.

4.14.2 Behavioral parameters Study

4.14.2.1 Anxiety tests

4.14.2.1.1 Elevated plus maze (EPM)

EPM is a widely used test for studying anxiolytic responses in rats. Rats have an aversion for open and high space and prefer to live in enclosed arm, when a rat is exposed to open arm there is a fear like movements and they freeze. The model is elevated at a height of 50cm consists of a central platform with two open arms crossed with two closed arms. Rat was placed separately in the central compartment with head facing towards open arm. The parameters that was evaluated were (a) Time spent and number of entries in the open arms b) Time spent and the number of entries was counted in the closed arms with four paws) were recorded for the period of five minutes [235].

4.14.2.1.2 Light and dark model

The model comprises of two plexiglass compartments one light (30×30×35 cm; 100 lx illumination) and one dark (20×30×35 cm; 40 lx illumination) connected by an opening of 7.5 × 7.5 cm in the middle of the divider. Rat was put separately in the middle of the light chamber having their back towards the dark compartment. The number of transitions between the light and dark compartment and the time spent in the light and dark chamber was recorded for five minutes [236].

4.14.2.2 Depression tests

4.14.2.2.1 Tail suspension test

Tail suspension test is a reliable and widely used method for screening antidepressant potential of the drugs. The test is designed for measuring the immobility time that is the indicator of change in mood. In tail suspension test rodents were hung from the 2-3 cm from the end of the tail by adhesive tape on the corner of a table 58 cm above the floor, in such a position that it cannot escape or hold the nearby surface. The duration of fixed status was

noted for a period of 5 min. The animals were viewed as immobile when they remain hanging passively and motionless [237].

4.14.2.2.2 Forced swim test

Forced swim test is a well-known and widely used test for evaluation of antidepressant drugs. In this test susceptibility of rats to the threat of drowning and is used to check the effectiveness of antidepressant drugs. In this rats were individually exposed to a situation of forced swimming, in an inescapable transparent vertical glass tank (height 45 cm, diameter 20cm) filled with water (temperature 27°C) to a depth of 20 cm. In this test after a brief spell of vigorous struggling, animal become immobile by keeping their heads above the water. The duration of immobility of the animal (in sec) was measured for 6 min. 'Immobility' of the rat was judged if it remains floating motionless, showing only those movements in which the head above water. This posture reflects a state of depression. The water was changed for each animal [238].

4.14.2.3 Seizures test

4.14.2.3.1 Seizure scoring

On third day of withdrawal animals received sub convulsive dose of Pentylenetetrazol (30mg/kg i.p.) 1hr after the administration of the drugs and CMC. After 30 minutes of Pentylenetetrazol administration rats were put in plexiglass chamber for 5 minutes for seizure scoring [239]. The seizures were recorded as given in Table 4.6.

Table 4.6: Seizure Scoring

Score 0	No reaction
Score 1	Ear and facial jerking,
Score 2	Convulsive wave all through the body
Score 3	Myoclonic jerks,
Score 4	Clonic tonic spasms, transform over into side position
Score 5	Generalized clonic tonic seizures with loss of postural control
Score 6	Death

4.14.2.4 Locomotor activity test

4.14.2.4.1 Actophotometer

The locomotor behavior of the rats was accessed using actophotometer. In this animals were placed individually and the count was recorded when the animal cuts off light beam falling on the photocell is recorded digitally. The duration of the test was of 5 min. Counter was stopped after five min and the reading was noted. The rat was removed thereafter and the counter resets to zero. The same protocol was repeated for all rats [240].

4.14.3 Hematological and biochemical examination

For hematological examination blood was collected from the retro-orbital sinus of the rats on 18^{th} day in EDTA coated tubes. Hematological parameters like WBC, MON, NEU, LYM, EOS, RBC, HGB, ESR, MCH, platelet count and PCV were determined. Clotted blood tubes was centrifuged at 3000 RPM and 4°C temperature for 10 min and the acquired serum was utilized to access biochemical parameters like total bilirubin, SGOT ,SGPT, albumin, globulin, Gamma-glutamyltransferase (GGT), blood urea, serum creatinine and serum uric acid through commercially available kits.

4.14.4 RT-PCR analysis

On completion of the study protocol rats were sacrificed by cervical dislocation method. The prefrontal cortex, amygdala and hippocampus were extracted from the whole brain using an adult rat brain matrix (Kent scientific, USA) and placed in a sterile tube containing RNAlater solution (5 volumes). The tubes containing the brain tissues were further stored at -80^{o}C until further analysis [241]. RT-PCR was carried out for studying mRNA expression of $GABA_A$ receptor subunits namely Gabra (1,2,3,4 and 5) and NMDA receptor subunits (Grin1, Grin2a, Grin2b). Trizol reagent was used for extracting total RNA from the brain tissues. RNA purity was evaluated on agarose gel (1.5%) in gel electrophoresis further quantification was done using a Nanodrop spectrophotometer (Thermo Scientific). Further 2 µg of total RNA was used for reverse transcription utilizing a verso RNA-to-cDNA synthesis kit (Thermo scientific). The RT-PCR analysis was performed using predesigned gene-specific primers for GABA and NMDA subunits using Primer Quest Tool (Table 4.7). Bio-Rad CFX96™ RT-PCR detection system was used for RT-PCR amplification using SYBR green dye. Total reaction mixer volume was 12.5 µl comprising of 2.5 pM of each primer and cDNA template (1 µl). Relative expression levels of the target genes were estimated using housekeeping gene

rat Beta-actin as an endogenous control. The thermal cycle profile for 40 cycles to amplify cDNA was as: 95 °C for 3 min; 95 °C for 15 s, 50 °C – 57 °C for 60 s and 72 °C for 2 min. The gene expression of the targeted genes was calculated by using double delta threshold cycle (ΔΔCT) method [242,243].

Table 4.7: Genes and Primer sequences used for RT-PCR study.

Sr. No.	Gene	Primer Sequence
1.	Gabra1	FP: GCCCTCCCAAGATGAACTTA RP: AGTTACACGCTCTCCCAAGC
2.	Gabra2	FP: ACCTTCTTTCACAACGGGAA RP: GGAAAGTCCTCCAAGTGCAT
3.	Gabra3	FP: CAAGCATGCCCCAGATATTC RP: CAGTCACTGCATCTCCAAGC
4.	Gabra4	FP: CCGTATCCTGGACAGTTTGC RP: ACATCAGAAACGGGTCCAAA
5.	Gabra5	FP: TGAGACCAATGACAACATCA RP: TAGATGTCTGTTCGCACCTG
6.	Grin1	FP: TTCAAGAGGGTGCTGATGTC RP: CCATTGTAGATGCCCACTTG
7.	Grin2a	FP: GTGATCGTGCTGAACAAGGA RP: CTGAGGTGGTTGTCATCTGG
8.	Grin2b	FP: CCGAAGCTGGTGATAATCCT RP: TCAGATGGTCATCCTCTTGC

4.15 Rat brain stereotaxic section coordinates relative to bregma (mm)

Isolated brain was placed in Adult Rat Brain Slicer Matrix with 500 micron section slice intervals (Coronal). The prefrontal cortex, amygdala and hippocampus were extracted from the whole rat brain using stereotaxic section coordinates. The selection of the coordinated is shown in the Fig 4.2 [244].

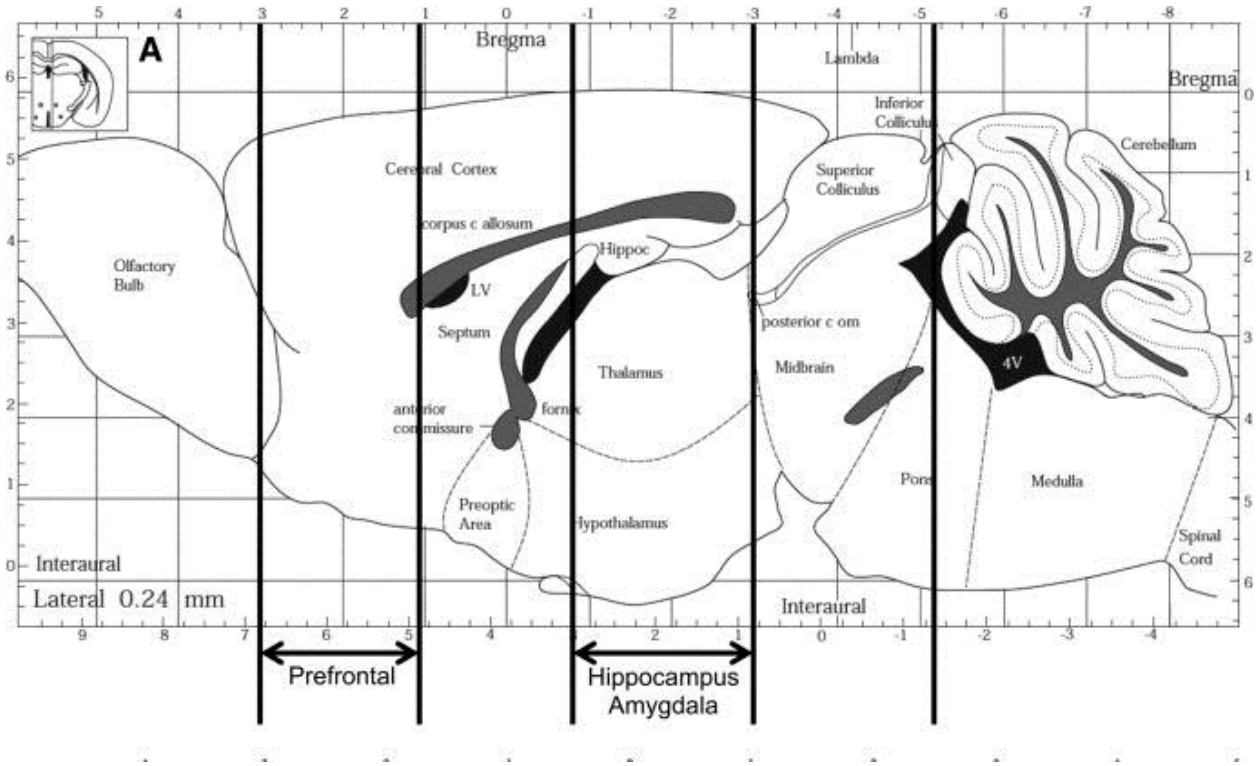


Fig 4.2: Rat brain stereotaxic section coordinates for Amygdala, Hippocampus and Prefrontal cortex relative to Bregma (mm).

4.16 Statistical analysis

GraphPad Prism software 8.0 was used for statistically scrutinizing the data and was expressed as the mean ± SEM (standard error of mean). Two way ANOVA was used for analyzing the data followed by Dunnett's multiple comparison post hoc test with a confidence level of $p<0.05$.

CHAPTER 5

RESULTS

5.1 Determination of extractable matter from the plants

All the six plants (*Bacopa monnieri, Hypericum perforatum, Centella asiatica, Withania somnifera, Camellia sinesis and Ocimum sanctum*) were subjected to the soxhlet extraction using hydroalcoholic solvents. The percentage yield obtained after the extraction of all the plants are presented in the Table 5.1.

Table 5.1: The percentage yield obtained after the extraction of the plant material**.**

Sr. No.	Plant	Dried Weight of Plant Material	Dried Extract Obtained	%Yield
1.	*Bacopa monnieri*	40g	5.5g	13.75
2.	*Hypericum perforatum*	40g	5.3g	13.25
3.	*Centella asiatica*	40g	5.8g	14.5
4.	*Withania somnifera*	40g	4.5g	11.25
5.	*Camellia sinensis*	40g	5.4g	13.5
6.	*Ocimum sanctum*	40g	6.4g	16

5.2 Phytochemical screening of the individual plant extracts

The presence of the major classes of phytoconstituents was confirmed by the qualitative phytochemical analysis of individual plant extracts which revealed the presence of variety of constituents like flavonoids, alkaloids, steroids & triterpenoids, saponin glycosides, glycosides, coumarin glycosides, anthraquinone glycosides, tannin and phenolic, amino acids, carbohydrates and pentoses etc. The results of phytochemical screening are represented in Table 5.2.

Table 5.2: Phytochemical screening of the individual plant extracts

Compound	*Bacopa monnieri*	*Hypericum perforatum*	*Centella asiatica*	*Withania somnifera*	*Camellia sinensis*	*Ocimum sanctum*
Flavonoids						
Shinoda test	+	+	+	+	+	+
Zinc hydrochloride test	+	+	+	+	+	+
Alkaloids						
Dragendroff's reagent	+	+	+	+	+	+
Hager's reagent	+	+	+	+	+	+
Mayer's reagent	+	+	+	+	+	+
Wagner's reagent	+	+	+	+	+	+
Phenolic and Tannins						
Ferric Chloride Test	+	+	+	-	+	+
Vanillin hydrochloride test	+	+	+	-	+	+
Steroids & triterpenoids						
Salkowski Test	+	+	+	+	-	+
Libermann-Burchard test	+	+	+	+	-	+
Glycosides						
Fehling's test	+	+	+	+	+	-
Saponin glycosides						
Froth test	+	+	+	+	-	-
Coumarin glycosides						
Coumarin Glycoside	+	-	+	+	-	-
Anthraquinone glycosides						

Borntrager's test	-	+	+	+	-	-
Cardiac Glycosides						
Keller Killian test	+	-	+	+	-	+
Amino acids						
Millon's test	-	+	+	+	+	+
Ninhydrin test	-	+	+	+	+	+
Carbohydrates						
Molisch's test	+	+	-	+	+	+
Pentoses						
Barfoed Test	+	+	-	+	+	+

Presence (+), Absence (-)

5.3 POL-6 preparation

Air dried powdered extract of plants (*Bacopa monnieri, Hypericum perforatum, Centella asiatica, Withania somnifera, Camellia sinesis and Ocimum sanctum*) was weighed independently and mixed in 2:1:2:2:1:2 proportions respectively utilizing a twofold cone blender. Weight of each plant extract used for POL-6 preparation is depicted in the Table 5.3.

Table 5.3: Ratios of the plant extracts used for the preparation of POL-6.

Sr. No.	Plant	Ratio	Weight in grams for 100 g
1.	*Bacopa monnieri*	2	20 g
2.	*Hypericum perforatum*	1	10 g
3.	*Centella asiatica*	2	20 g
4.	*Withania somnifera*	2	20 g
5.	*Camellia sinensis*	1	10 g
6.	*Ocimum sanctum*	2	20 g

5.4 Organoleptic assessment of POL-6

Physical and chemical parameters of the prepared POL-6 were tested as per Indian Pharmacopoeia standards. Evaluation of physical parameters showed that POL-6 is a dark brown colored powder having fine texture, pungent odor and bitter taste.

5.5 Physiochemical investigation and solvent extractive values of Pol-6

As a part of standardization, quality tests were performed on POL-6 for Loss on Drying at 105ºC (4.12 ± 0.121), Acid insoluble ash (%) (0.62 ± 0.012), Total ash (%) (15.30 ± 0.023), pH (1% aq. solution) (7.14 ± 0.217), Alcoholic soluble extractive value (%) (69.48 ± 0.485), Water soluble extractive value (%) (46.14 ± 0.213). The obtained results from the physiochemical investigation and solvent extractive vales of POL-6 were found within Indian Pharmacopoeia standard limits (Table 5.4).

Table 5.4: Physiochemical characteristics of POL-6.

S.no.	Parameters (I.P Limit values)	Percentage Mean
1.	Loss on Drying 105ºC (%) (<5)	4.12 ± 0.121
2.	Water soluble Extractive Value (%)	46.14 ± 0.213
3.	Alcoholic Soluble Extractive Value (%)	69.48 ± 0.485
4.	Total Ash (%) (<15)	15.30 ± 0.023
5.	Acid Insoluble Ash (%) (<5)	0.62 ± 0.012
6.	pH	7.14 ± 0.217

The results were expressed as mean (n=3) ± Standard deviation (SD)

5.6 Physical characteristics of POL-6

The results of physical properties of POL-6 like Bulk density 0.441 ± 0.001 (g/ml), Tap density 0.612 ± 0.011 (g/ml), Hausner's ratio 1.38 ± 0.2, Carr's index 38.77 % and Angle of repose 27.2 ± 0.3 are depicted in Table 5.5.

Table 5.5: Physical characteristics of POL-6.

S. no.	Parameters	Results	Standard I.P limits
1	Bulk Density	0.441 ± 0.001 (g/ml)	Good flow (<1)
2	Tapped Density	0.612 ± 0.011 (g/ml)	Good flow (<1)
3	Carr's Index	38.77 %	Poor (>38)
4	Hausner's Ratio	1.38 ± 0.2	Possible (1.26 – 1.38)
5	Angle of Repose	27.2 ± 0.3	Good (25-30)

5.7 Phytochemical screening of POL-6

The qualitative phytochemical analysis of POL-6 revealed the presence of flavonoids, alkaloids, steroids & triterpenoids, glycosides, saponin glycosides, coumarin glycosides, anthraquinone glycosides, tannin and phenolic, amino acids, carbohydrates and pentoses (Table 5.6).

Table 5.6: Qualitative phytochemical analysis of POL-6.

Compound	Detection Method	Inference
Flavonoids	Shinoda test	+
	Zinc hydrochloride test	+
Alkaloids	Dragendroff's reagent	+
	Hager's reagent	+
	Mayer's reagent	+
	Wagner's reagent	+
Phenolic and Tannins	Ferric Chloride Test	+
	Vanillin hydrochloride test	+
Steroids & triterpenoids	Salkowski Test	+
	Libermann-Burchard test	+
Glycosides	Fehling's test	+
Saponin glycosides	Froth test	+
Coumarin glycosides	Coumarin Glycoside	+
Anthraquinone glycosides	Borntrager's test	+
Cardiac Glycosides	Keller Killian test	+
Amino acids	Millon's test	+
	Ninhydrin test	+
Carbohydrates	Molisch's test	+
Pentoses	Barfoed Test	+

Presence (+), Absence (-)

5.8 Quantification of total phenolic, flavonoid and tannin content in POL-6

Phytoconstituents confirmed by qualitative phytochemical screening were further quantified by quantitative phytochemical analysis. Total phenolic content of POL-6 was calculated as mg of Gallic acid (GAE) equivalents/g dry weight (DW) (mg GAE/g DW) and was found to be 316.66±0.28 mg GAE/g. The total flavonoid content of POL-6 was calculated as mg of quercetin (QR) equivalents/g dry weight (mg QR/g DW) and was found to be 481.14±0.34 mg QR/g. Tannin content of POL-6 was calculated as mg of catechin (CE) equivalents/g dry weight (mg CE/g DW) and was found to be 100.43±0.41. The results of quantitative phytochemical screening are depicted in the Table 5.7.

Table 5.7: Quantitative analysis of phenolic, flavonoid and tannin content in Pol-6.

Polyphenol content (mg of GAE/g DW)	Flavonoid content (mg of QR/g DW)	Tannin content (mg of CE/g DW)
316.66±0.28	481.14±0.34	100.43±0.41

5.9 Qualitative phytochemical profiling of POL-6 by HPLC

Qualitative phytochemical of POL-6 was also performed by HPLC analysis. HPLC chromatogram showed eleven peaks indicating the presence of eleven major compounds. Different peaks, retention time, peak area and area percentage is shown in Fig 5.1, Table 5.8. The identification and quantifications of the compounds was further studied through LC-MS and HPTLC.

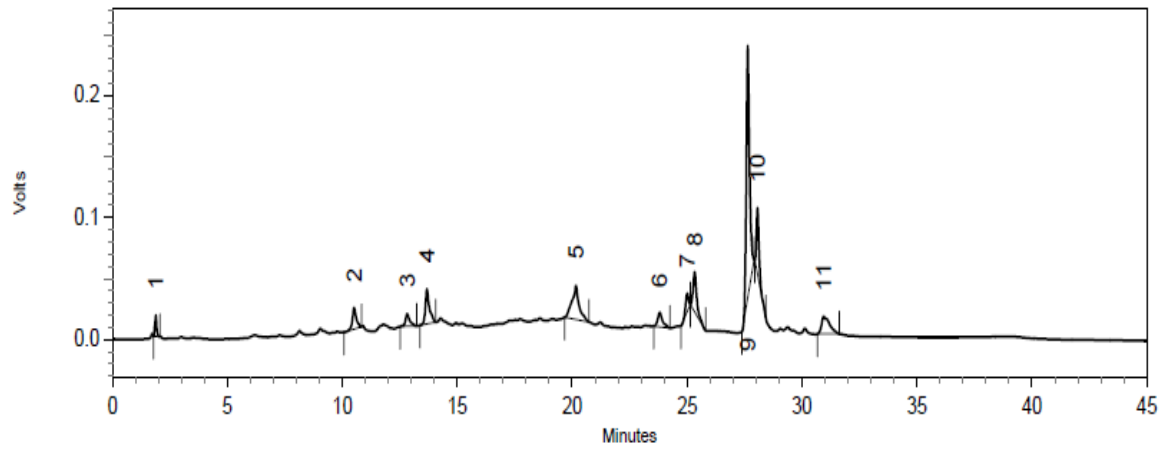


Fig 5.1: HPLC Chromatogram of POL-6.

Table 5.8: HPLC retention time and peak area indicating the presence of 11 major compounds.

Sr. No.	Retention Time	Area	Percentage of total area
1.	1.892	90417	1.90
2.	10.517	201504	4.23
3.	12.825	136234	2.86
4.	13.683	343799	7.22
5.	20.167	593571	12.46
6.	23.808	171106	3.59
7.	25.008	118638	2.49
8.	25.325	352297	7.39
9.	27.642	1990010	41.77
10.	28.058	417285	8.76
11.	30.942	349156	7.33

5.10 Liquid chromatography-mass spectroscopy profiling of POL-6

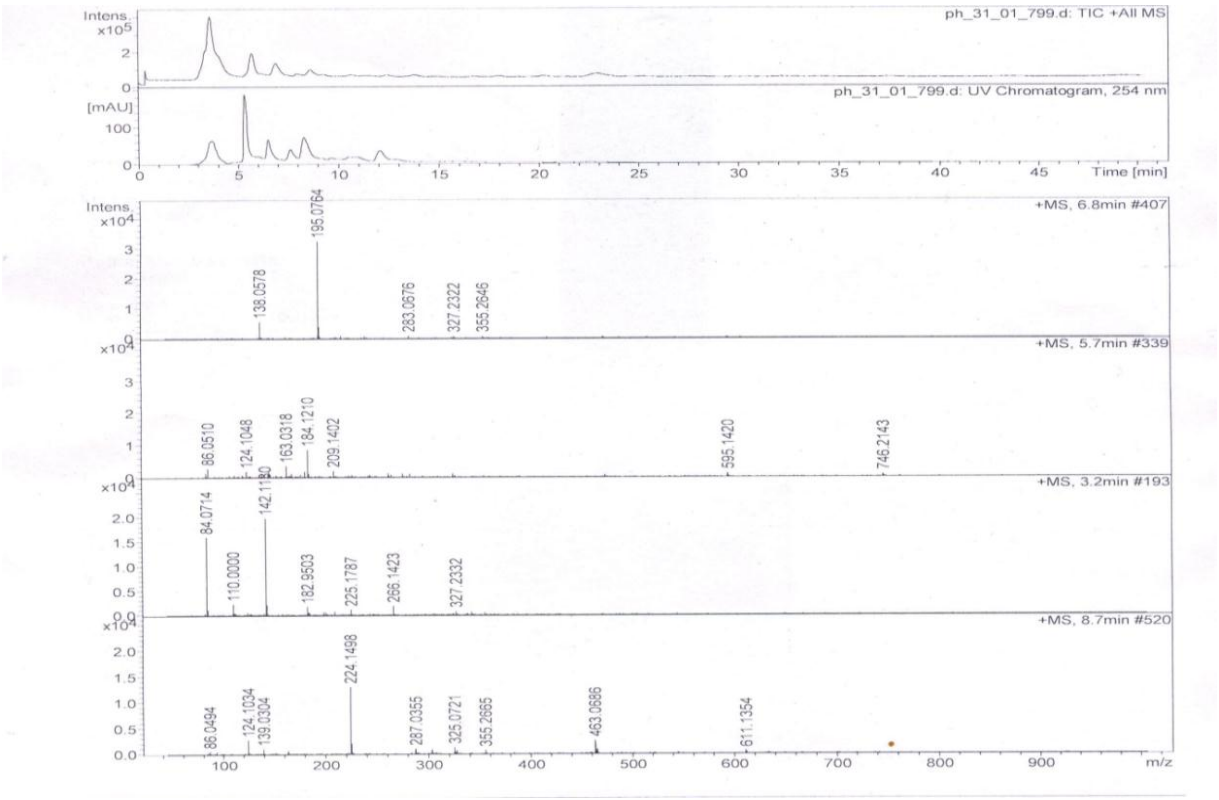


Fig 5.2: LC-MS spectrum of POL-6 showing peaks and molecular mass of different compounds (Appendix C).

Mass calculated for $C_{28}H_{38}O_6$ (Withaferin A) exact mass 470.606 g/mol, fragmentation pattern ((steroid –OH) + H^+), found mass 287.0355; $C_{28}H_{38}O_6$ (Withanolide) exact mass 470.606 g/mol, fragmentation pattern ((steroid –OH) + H^+), found mass 287.0355; $C_{15}H_{10}O_6$ (Luteolin) exact mass 286.24 g/mol, fragmentation pattern (+ H^+), found mass 287.0355; $C_{29}H_{50}O$ (β-Setosterol) exact mass 414.71 g/mol, fragmentation pattern (Isopropyl- CH_3), found mass 287.0355; $C_{15}H_{10}O_7$ (Quercetin) exact mass 302.236 g/mol, fragmentation pattern (+ Na^+), found mass 325.0721; $C_{41}H_{68}O_{13}$, (Bacoside A) exact mass 769 g/mol, fragmentation pattern (-sugar + Na^+), found mass 463.0686; $C_{30}H_{16}O_8$ (Hypericin) exact mass 504.44 g/mol, fragmentation pattern (-2Ketone,-OH, -Anthracene one connection), found mass 464.01; $C_{27}H_{30}O_{16}$ (Rutin) exact mass 610.52 g/mol, fragmentation pattern (+H^+), found mass 611.1354; $C_{10}H_{18}O$ (Linalool) exact mass 154.25 g/mol, fragmentation pattern (-CH_2), found mass 142.11; $C_9H_8O_4$ (Caffeic acid) exact mass 181.16 g/mol, fragmentation pattern (+H^+), found mass 182.9503; $C_{15}H_{14}O_6$ (Catechin) exact mass 290.26 g/mol, fragmentation pattern (-O-hydroxyphenol), found mass 182.9503; $C_{10}H_{12}O_2$ (Eugenol) exact mass 164.2 g/mol, fragmentation pattern (-H^+), found mass 163.0318; $C_6H_{14}O_6$ (D-Mannitol) exact mass 182.172 g/mol, fragmentation pattern (+H^+), found mass 184.1210; $C_{28}H_{38}O_6$ (Withanone) exact mass 470.6 g/mol, fragmentation pattern (-Pyran, -CH_3), found mass 327.22 Fig 5.2, Fig. 5.3. Identified compounds and their expected and observed molecular mass are given in the Table 5.9.

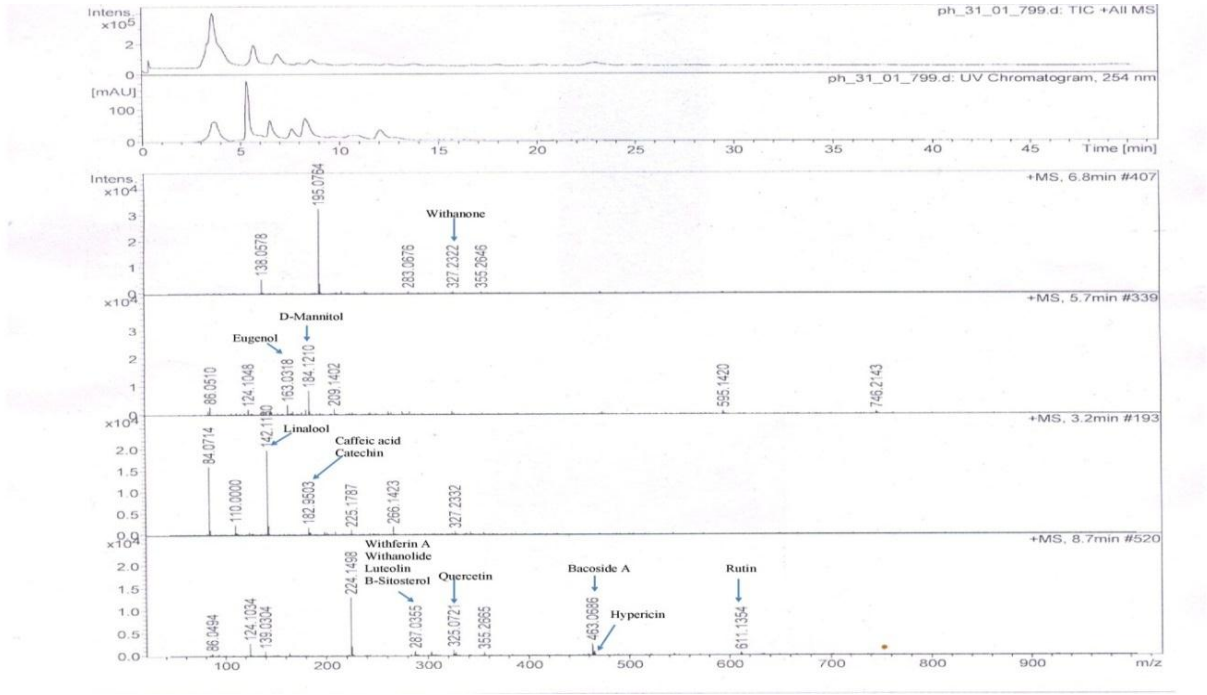


Fig 5.3: LC-MS spectrum of POL-6 showing the major identified compounds.

Table 5.9: Identified compounds and their expected and observed fragmented and total molecular mass.

Sr. No.	Compound As In Spectra	Expected Molecular Mass (g/mol)	Observed Mass of the Fragment/Total Molecule (g/mol)
1.	Withaferin A	470.606	287.0355 (Fragmented Mass)
2.	Withanolide	470.606	287.0355 (Fragmented Mass)
3.	Luteolin+ H^+	287.24	287.0355 (Total Molecule)
4.	β-Setosterol	414.71	287.0355 (Fragmented Mass)
5.	Quercetin + Na^+	325.236	325.0721 (Total Molecule)
6.	Bacoside A	769	463.0686 (Fragmented Mass)
7.	Hypericin	504.44	464.01 (Fragmented Mass)
8.	Rutin+ H^+	611.52	611.1354 (Total Molecule)
9.	Linalool	154.25	142.11 (Fragmented Mass)
10.	Caffeic acid+ H^+	182.16	182.9503 (Total Molecule)
11.	Catechin	290.26	182.9503 (Fragmented Mass)
12.	Eugenol-H^+	163.2	163.0318 (Total Molecule)
13.	D-Mannitol+ H^+	184.172	184.1210 (Total Molecule)
14.	Withanone	470.6	327.22 (Fragmented Mass)

5.11 Quantification of major constituents present in POL-6 by HPTLC

Major Phytoconstituents identified by LC-MS was further quantified by the HPTLC. Quantification of six compounds Withaferin, Quercetin, Caffeic acid, β-Sitosterol, Rutin and Catechin was performed by HPTLC (Appendix A).

5.11.1 Quantification of Withaferin A in POL-6 by HPTLC

Mobile phase consisting chloroform: methanol (9.5:0.5, v/v) showed sharp peaks with Rf value of 0.59 for Withaferin A (Fig 5.4 A). Withferin A found in the POL-6 was 0.921% w/w of POL-6. HPTLC chromatogram of POL-6 and Withaferin A is presented in the Fig 5.4 B.

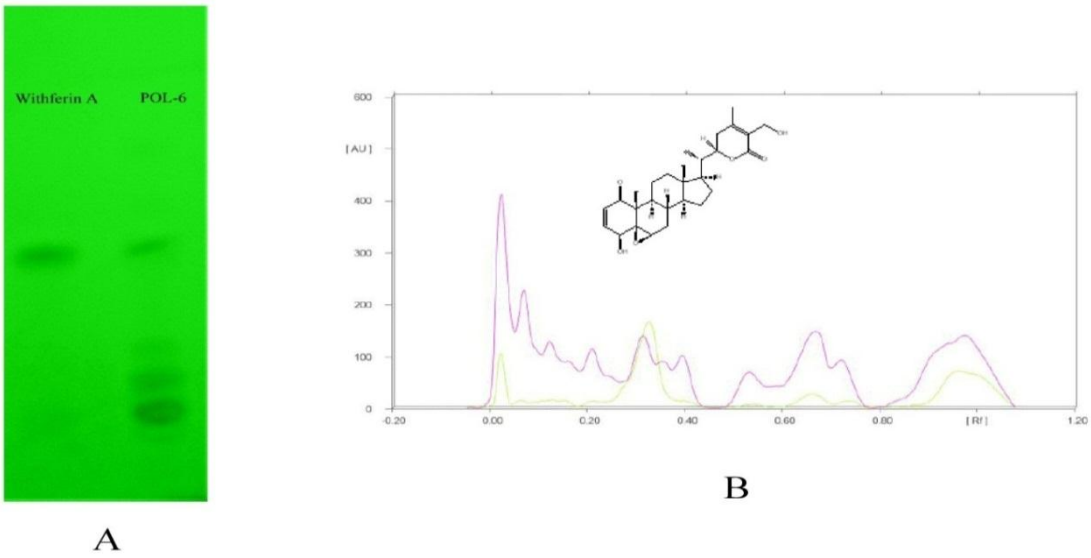


Figure 5.4: TLC and HPTLC estimation of Withaferin A in POL-6. (A) TLC plate of POL-6 with Withaferin A (B) HPTLC chromatogram of POL-6 and Withaferin A.

5.11.2 Quantification of Quercetin in POL-6 by HPTLC

Mobile phase consisting Toluene: methanol (7:3, v/v) showed sharp peaks with Rf value of 0.65 for Quercetin (Fig 5.5 A). Quercetin found in the POL-6 was 1.50% w/w of POL-6. HPTLC chromatogram of POL-6 and quercetin is presented in the Fig 5.5 B.

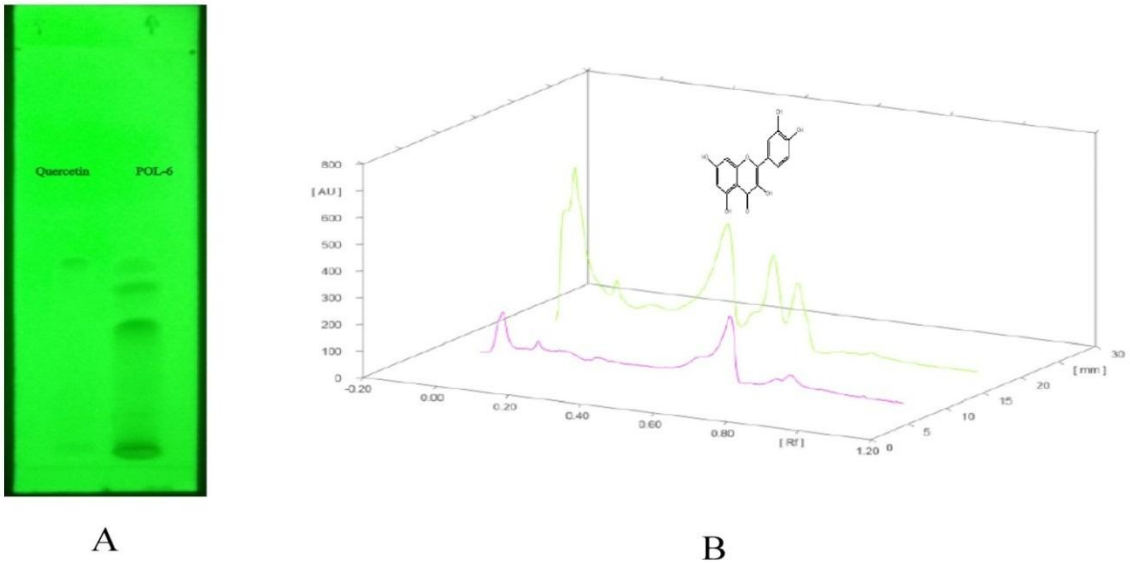


Figure 5.5: TLC and HPTLC estimation of quercetin in POL-6. (A) TLC plate of POL-6 with Quercetin (B) HPTLC chromatogram of POL-6 and quercetin.

5.11.3 Quantification of Caffeic acid in POL-6 by HPTLC

Mobile phase consisting toluene: ethyl acetate: formic acid (5:4:1, v/v/v) revealed sharp peaks with Rf value of 0.49 for Caffeic acid (Fig 5.6 A). Caffeic acid found in the POL-6 was 1.059% w/w of POL-6. HPTLC chromatogram of POL-6 and Caffeic acid is presented in the Fig 5.6 B.

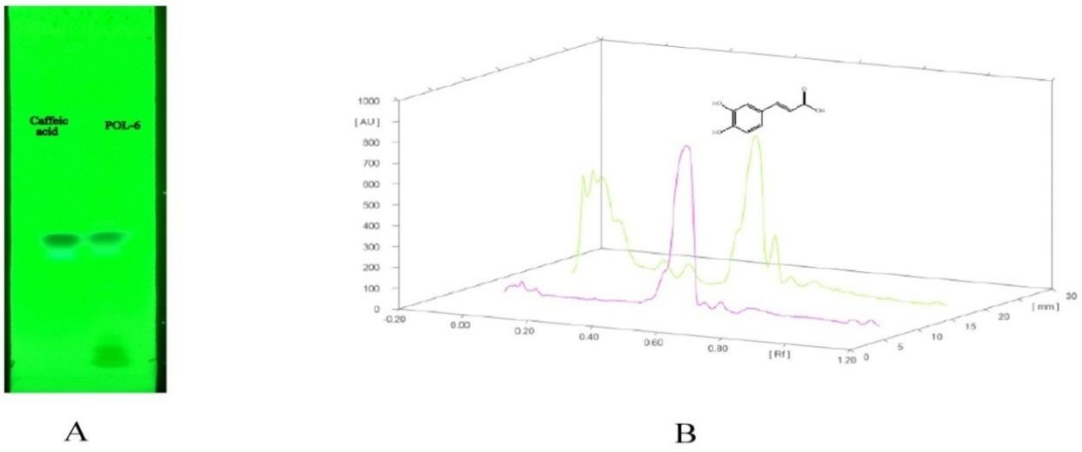


Figure 5.6: TLC and HPTLC estimation of Caffeic acid in POL-6. (A) TLC plate of POL-6 with Caffeic acid (B) HPTLC chromatogram of POL-6 and Caffeic acid.

5.11.4 Quantification of Rutin in POL-6 by HPTLC

Mobile phase consisting ethyl acetate: formic acid: acetic acid: water (10:1.1:1.1:2.6, v/v/v/v) showed sharp peaks with Rf value of 0.86 for Rutin (Fig 5.7 A). Rutin found in the POL-6 was 0.86% w/w of POL-6. HPTLC chromatogram of POL-6 and Rutin is presented in the Fig 5.7 B.

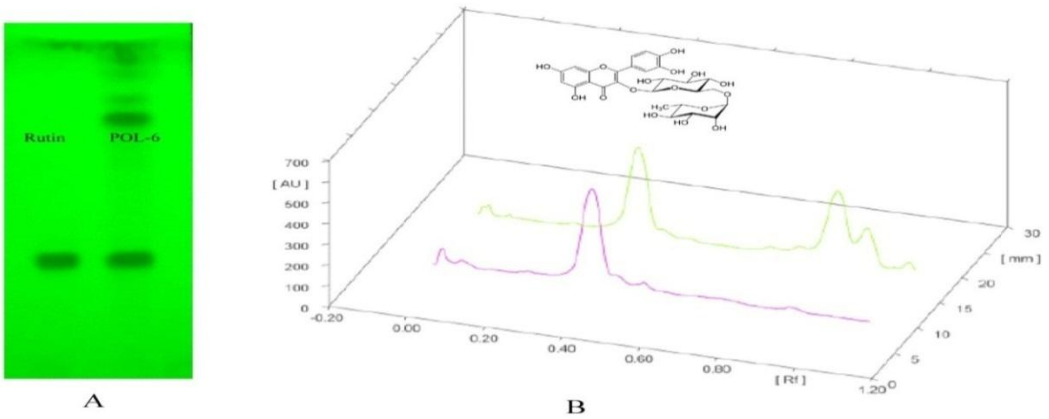


Figure 5.7: TLC and HPTLC estimation of Rutin in POL-6. (A) TLC plate of POL-6 with Rutin (B) HPTLC chromatogram of POL-6 and Rutin.

5.11.5 Quantification of β-Sitosterol in POL-6 by HPTLC

Mobile phase consisting n-hexane: ethyl acetate (8:2, v/v) showed sharp peaks with Rf value of 0.61 for β-Sitosterol (Fig 5.8 A). β-Sitosterol found in the POL-6 was 0.60% w/w of POL-6. HPTLC chromatogram of POL-6 and β-Sitosterol is presented in the Fig 5.8 B.

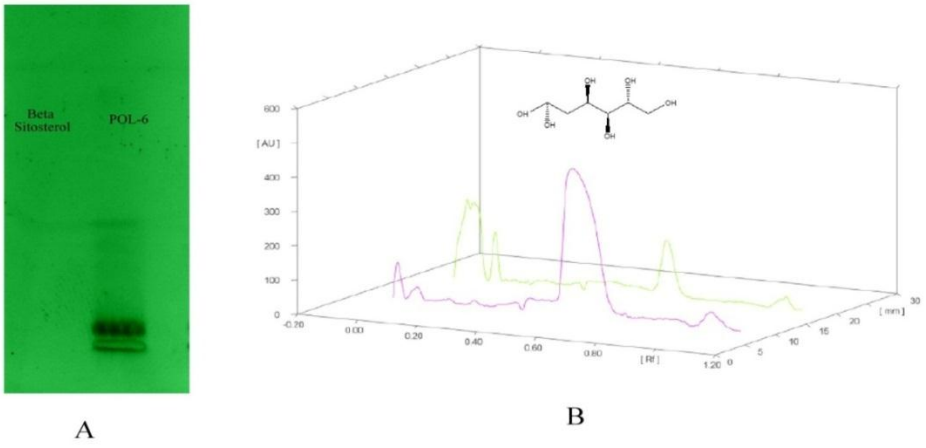


Figure 5.8: TLC and HPTLC estimation of β-Sitosterol in POL-6. (A) TLC plate of POL-6 with β-Sitosterol (B) HPTLC chromatogram of POL-6 and β-Sitosterol.

5.11.6 Quantification of Catechin in POL-6 by HPTLC

Mobile phase consisting toluene: ethyl acetate: formic acid (5:4:1, v/v/v) revealed sharp peaks with Rf value of 0.22 for Catechin (Fig 5.9 A). Catechin found in the POL-6 was 2.86% w/w of POL-6. HPTLC chromatogram of POL-6 and Catechin is presented in the Fig 5.9 B.

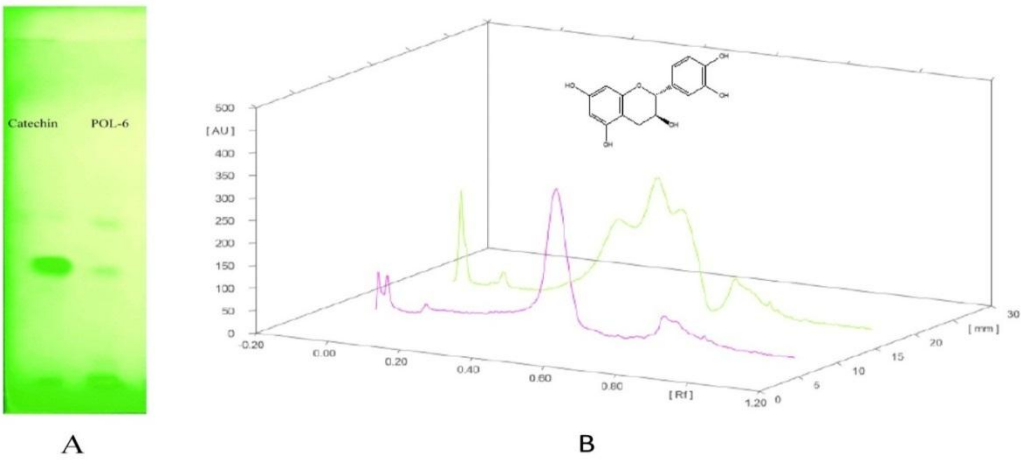


Figure 5.9: TLC and HPTLC estimation of Catechin in POL-6. (A) TLC plate of POL-6 with Catechin (B) HPTLC chromatogram of POL-6 and Catechin.

5.12 Evaluation of free radical scavenging activity of POL-6

5.12.1 DPPH radical scavenging assay

DPPH assay is based upon the fact that the presence of antioxidant molecules in test sample scavenges DPPH radicals and forms yellow colored compound di phenyl hydrazine. Free radical (DPPH) scavenging activity of POL-6 was compared with standard ascorbic acid. The standard curve of ascorbic acid was prepared and depicted in the Fig 5.10. The IC_{50} values were determined from the percentage inhibitory concentrations and were found to be POL-6 (22.54±0.21 µg/ml) and ascorbic acid (18±0.18 µg/ml). The obtained results are depicted in Table 5.10.

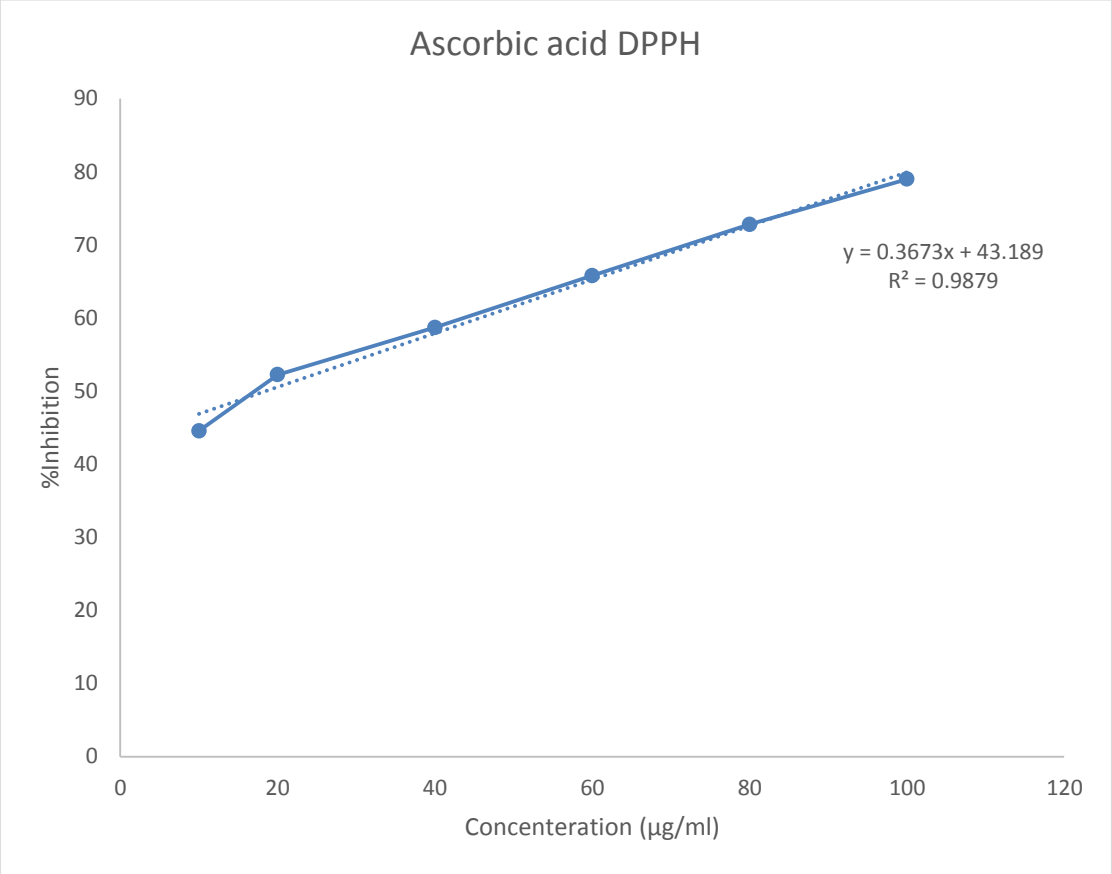


Fig 5.10: Ascorbic acid standard curve by DPPH method.

5.12.2 Nitric oxide radical scavenging activity

The free radical scavenging activity of POL-6 against nitric oxide formation was compared with ascorbic acid (standard). The standard curve of ascorbic acid was prepared and depicted in Fig 5.11. The IC_{50} values were calculated from the percentage inhibitory concentrations

and found to be POL-6 (21.38±0.17 µg/ml) and Ascorbic acid (17.99 ± 0.17 µg/ml). The obtained results are depicted in Table 5.10.

Table 5.10: Free radical scavenging activity of POL-6

Sample Name	$IC_{50/DPPH}$ (µg/ml)	$IC_{50/NO2}$ (µg/ml)
POL-6	22.54±0.21	21.38±0.13
Ascorbic acid	18 ± 0.18	17.99±0.17

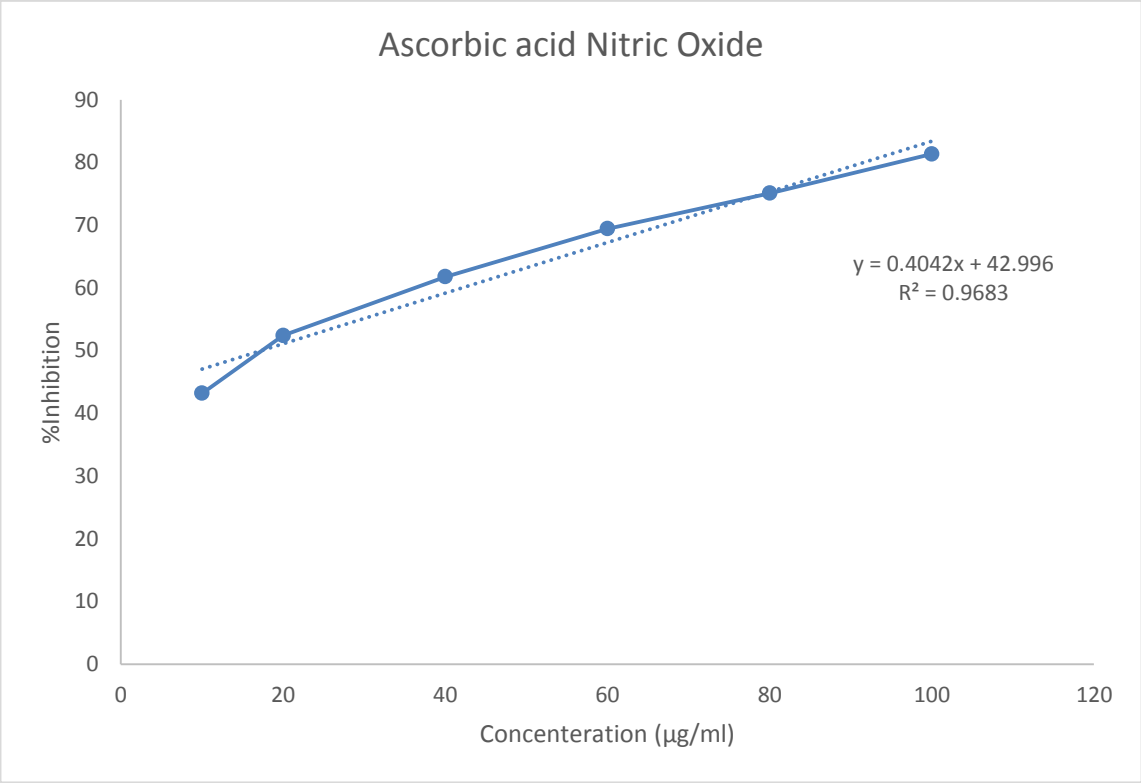


Fig 5.11: Standard curve of ascorbic acid by nitric oxide assay method.

5.13 Acute study

In acute toxicity evaluation, no behavioral signs of toxicity were observed in the rats after single administration of POL-6 (2000 mg/kg) orally. POL-6 administration did not showed any signs of general activity (conscious state), corneal reflexes, muscle coordination, tremors, straub, convulsions, anesthesia, sedation, piloerection, lacrimation, ptosis and salivation in rats. Normal control group rats and POL-6 treated rats were similar in clinical manifestation**.** POL-6 did not induce any changes in water and food intake in rats (Table 5.11). POL-6

treated rats did not showed any changes in relative organ weights when compared to the control group rats (Table 5.12). No treatment-related gross pathological changes were seen during the organ's necropsy examination and all rats survived during the 14 day study period (Fig 5.12).

Table 5.11: Body weight changes, food and water intake of rats treated orally with POL-6 in acute toxicity study. Values expressed as mean ± SEM, n = 5 animals/group. $p<0.05$ (ANOVA/Dunnett's test) compared with respective control group.

Parameter	Control Group	Treatment Group (2000mg/kg)
Initial weight (g)	190.60 ± 3.08	180.70 ± 5.11
Final weight (g)	208.30 ± 6.02	198.60 ± 6.82
Body weight gain (%)	9.25 ± 3.02	9.62 ± 3.21
Food intake (g/day)	20.05 ± 3.08	22.32 ± 5.74
Water intake (ml/day)	23.60 ± 4.08	24.09 ± 9.10

Table 5.12: Relative organ weight (%) of rats treated orally with POL-6. Values expressed as mean ± SEM, n = 5 animals/group. $p<0.05$ (ANOVA/Dunnett's test) compared with respective control group. The relative organ weights of kidneys, testes, ovaries and adrenal glands mentioned are the combined weights of both the right and left organ.

Organ	Control Group	Treatment Group (2000mg/kg)
Brain	1.97 ± 0.08	1.70 ± 0.03
Heart	0.72 ± 0.01	0.71 ± 0.01
Liver	6.57 ± 0.40	6.62 ± 0.23
Spleen	0.51 ± 0.01	0.51 ± 0.00
Thymus	0.38 ± 0.01	0.33 ± 0.00
Kidney	1.87 ± 0.02	1.82 ± 0.04
Adrenals	0.04 ± 0.00	0.04 ± 0.00
Ovaries	0.12 ± 0.00	0.11 ± 0.00
Uterus	0.55 ± 0.01	0.53 ± 0.03

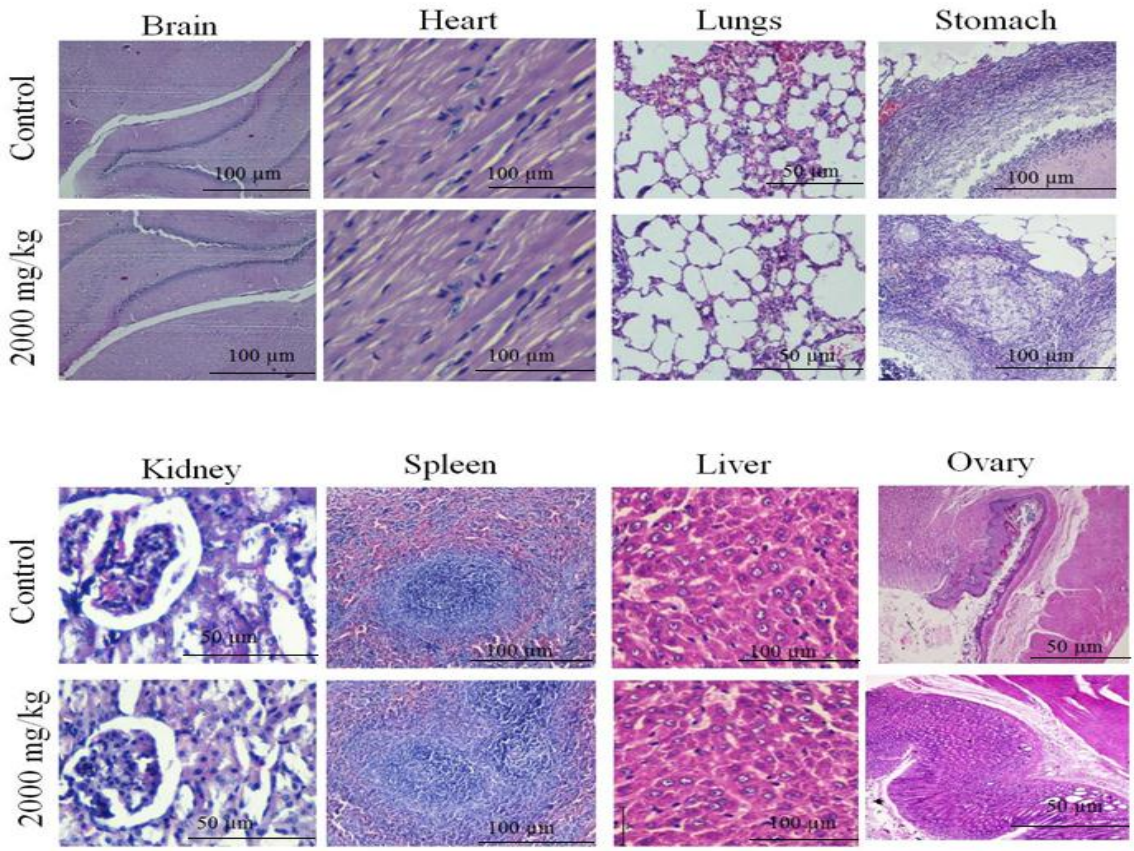


Fig 5.12. Histology of different organs treated with POL-6 in the acute toxicity study.

5.14 Sub-acute study

5.14.1 Effects on the general behavior and mortality

In sub-acute toxicity of 28 days repeated dose study, POL-6 (250, 500 and 1000 mg/kg) treated animals did not showed any behavioral signs of general activity (conscious state), corneal reflexes, tremors, muscle coordination, straub, convulsions, anesthesia, sedation, lacrimation, ptosis, piloerection and salivation when compared to the control group animals. No mortality was noticed during the study period.

5.14.2 Effects on the body weight, feed, and water intake

No significant changes in the body weights, feed consumption and water intake were observed in the POL-6 treated animals of both sexes when compared to the control group animals. (Table 5.13).

Table 5.13: Body weight changes, food and water intake of rats treated orally with POL-6 in sub-acute toxicity. Values expressed as mean ± SEM, n = 5 animals/group. $p<0.05$ (ANOVA/Dunnett's test) compared with respective control group

Parameter	Control	250 mg/kg	500 mg/kg	1000 mg/kg	High Dose Satellite
Male Rats					
Initial weight (g)	228.34 ± 5.09	220.03 ± 5.98	231.92 ± 6.01	230.63 ± 4.34	235.97 ± 6.75
Final weight (g)	261.30 ± 6.03	259.09 ± 11.09	270.01 ± 3.21	269.82 ± 5.71	269.41 ± 5.04
Body weight gain (%)	16.05 ± 4.05	18.03 ± 5.21	19.05 ± 4.16	18.49 ± 7.02	17.07 ± 7.03
Food intake (g/day)	21.92 ± 5.08	22.34 ± 4.10	20.91 ± 5.86	24.14 ± 3.25	20.02 ± 6.84
Water intake (ml/day)	20.16 ± 6.32	21.07 ± 8.03	24.08 ± 4.18	23.46 ± 5.78	21.49 ± 7.19
Female Rats					
Initial weight (g)	181.08 ± 3.91	179.82 ± 3.89	180.29 ± 4.39	179.98 ± 3.12	182.20 ± 4.61
Final weight (g)	218.70 ± 6.42	214.10 ± 7.80	218.60 ± 8.90	213.34 ± 5.05	222.09 ± 7.08
Body weight gain (%)	19.83 ± 5.16	18.02 ± 5.13	19.01 ± 7.05	18.97 ± 6.15	19.94 ± 4.17
Food intake (g/day)	23.06 ± 4.94	21.88 ± 6.21	23.87 ± 6.02	21.89 ± 8.59	22.25 ± 6.41
Water intake (ml/day)	20.13 ± 4.76	23.03 ± 8.49	24.12 ± 9.01	24.10 ± 5.99	22.82 ± 7.02

5.14.3 Haemato–biochemical parameters

Hematological examination showed that POL-6 treated animals did not produce any significant changes as all analyzed hematological parameters of both sexes were in normal values when compared to the control group animals. The results of the hematological parameters of both sexes are depicted in the Table 5.14. POL-6 treatment did not produce any significant changes in the biochemical parameter analysis in both male and female rats when compared to the control group animals. The results of the biochemical parameters of both sexes are depicted in the Table 5.15.

Table 5.14: Hematological parameters of rats treated orally with POL-6. Values expressed as mean ± SEM, n = 5 animals/group. $p<0.05$ (ANOVA/Dunnett's test) compared with respective control group.

Parameter	Control	250 mg/kg	500 mg/kg	1000 mg/kg	High Dose Satellite
Male Rats					
WBC(10^3µL)	4.46 ± 1.09	4.58 ± 0.89	4.83 ± 1.02	5.15 ± 0.73	4.52 ± 0.92
LYM -R%	78.83 ± 1.07	79.0 ± 1.02	76.83 ± 0.81	77.23 ± 1.31	75.11 ± 1.03
MON-R %	1.22 ± 0.16	1.18 ± 0.13	1.16 ± 0.11	1.23 ± 0.11	1.29 ± 0.17
NEU-R %	17.50 ± 0.61	19.27 ± 0.50	18.29 ± 0.32	20.97 ± 0.40	17.95 ± 0.29
EOS-R %	1.00 ± 0.03	1.06 ± 0.18	1.02 ± 0.10	1.03 ± 0.19	1.01 ± 0.04
BAS-R %	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
RBC (10^6/µL)	5.11 ± 0.06	5.18 ± 0.06	4.98 ± 0.08	5.25 ± 0.06	5.10 ± 0.07
HGB (g/dL)	13.32 ± 0.04	12.92 ± 0.07	13.30 ± 0.09	13.42 ± 0.08	13.19 ± 0.09
MCV (µm^3)	88.07 ± 0.52	87.71 ± 0.38	85.98 ± 0.32	87.13 ± 0.05	88.17 ± 0.27
MCH (pg)	24.70 ± 0.53	23.82 ± 0.34	24.32 ± 0.38	25.05 ± 0.28	24.92 ± 0.63
PLT (10^3/µL)	2.24 ± 0.07	2.27 ± 0.09	2.94 ± 0.06	2.16 ± 0.05	2.28 ± 0.07
ESR (mm/1^{st}hr)	1.33 ± 0.21	1.26 ± 0.13	1.41 ± 0.11	1.29 ± 0.21	1.37 ± 0.15
PCV (%)	42.58 ± 0.90	42.10 ± 0.74	39.91 ± 0.54	42.09 ± 0.86	42.49 ± 0.71
Female Rats					
WBC(10^3µL)	4.41 ± 1.45	4.30 ± 1.16	4.51 ± 1.13	4.46 ± 1.01	4.48 ± 2.12
LYM -R%	57.14 ± 0.73	58.31 ± 0.71	61.23 ± 0.51	60.21 ± 0.81	59.23 ± 0.49
MON-R %	2.00 ± 0.36	2.31 ± 0.41	1.98 ± 0.57	2.21 ± 0.32	2.01 ± 0.23
NEU-R %	36.83 ± 1.07	32.80 ± 1.36	34.13 ± 1.23	35.14 ± 0.59	33.04 ± 1.21
EOS-R %	0.83 ± 0.30	0.74 ± 0.24	0.81 ± 0.12	0.79 ± 0.11	0.77 ± 0.03
BAS-R %	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
RBC (10^6/µL)	4.84 ± 0.07	4.71 ± 0.03	4.91 ± 0.08	4.86 ± 0.11	4.73 ± 0.09
HGB (g/dL)	12.70 ± 0.05	12.82 ± 0.09	12.90 ± 0.03	12.64 ± 0.08	12.72 ± 0.06
MCV (µm^3)	21.88 ± 0.32	21.72 ± 0.56	21.93 ± 0.44	20.92 ± 0.71	21.90 ± 0.32
MCH (pg)	79.63 ± 5.61	82.13 ± 4.12	78.12 ± 5.12	85.15 ± 3.14	81.24 ± 3.91
PLT (10^3/µL)	4.06 ± 0.02	3.98 ± 0.02	4.13 ± 0.02	3.82 ± 0.04	4.13 ± 0.03
ESR (mm/1^{st}hr)	1.50 ± 0.34	1.54 ± 0.21	1.46 ± 0.12	1.51 ± 0.18	1.53 ± 0.16
PCV (%)	42.32 ± 0.84	41.97 ± 0.35	41.08 ± 0.45	39.13 ± 0.23	40.87 ± 0.31

Table 5.15: Biochemical parameters of rats treated orally with POL-6. Values expressed as mean ± SEM, n = 5 animals/group. $p<0.05$ (ANOVA/Dunnett's test) compared with respective control group.

Parameter	Control	250 mg/kg	500 mg/kg	1000 mg/kg	High Dose Satellite
Male Rats					
ALB (g/L)	30.31 ± 0.02	30.71 ± 0.03	30.23 ± 0.05	30.43 ± 0.06	30.71 ± 0.09
CHOL (mg/dL)	90.03 ± 0.14	90.12 ± 0.07	90.28 ± 0.13	90.62 ± 0.09	90.24 ± 0.05
Serum Protein (g/L)	6.55 ± 0.07	6.47 ± 0.04	6.41 ± 0.09	6.59 ± 0.08	6.45 ± 0.06
ALP (U/L)	266.5 ± 0.16	242.12 ± 0.14	234.5 ± 0.12	254.12 ± 0.13	260.51 ± 0.11
CRE (mg/dL)	0.50 ± 0.01	0.51 ± 0.03	0.50 ± 0.04	0.50 ± 0.03	0.51 ± 0.01
Blood Urea (mg/dL)	21.86 ± 0.01	22.09 ± 0.06	20.87 ± 0.08	21.98 ± 0.07	22.53 ± 0.08
Total Bilirubin (mg/dL)	0.50 ± 0.02	0.51 ± 0.06	0.50 ± 0.01	0.49 ± 0.08	0.51 ± 0.02
Bilirubin (direct) (mg/DL)	0.21 ± 0.04	0.22 ± 0.03	0.20 ± 0.07	0.21 ± 0.06	0.22 ± 0.05
Bilirubin (indirect)	0.27 ± 0.06	0.26 ± 0.09	0.27 ± 0.03	0.28 ± 0.06	0.27 ± 0.05
SGOT (U/L)	87.50 ± 0.76	82.43 ± 0.55	79.00 ± 0.89	84.03 ± 0.72	82.99 ± 0.49
SGPT (U/L)	46.50 ± 0.76	44.90 ± 0.67	45.60 ± 0.51	43.74 ± 0.89	45.13 ± 0.71
Globulin (gm%)	3.46 ± 0.11	3.72 ± 0.06	3.45 ± 0.20	3.53 ± 0.09	3.48 ± 0.06
A/G Ratio (Ratio)	0.81 ± 0.06	0.80 ± 0.02	0.79 ± 0.01	0.82 ± 0.01	0.76 ± 0.04
Female Rats					
ALB (g/L)	30.34 ± 0.09	30.07 ± 0.11	30.21 ± 0.03	30.09 ± 0.12	30.23 ± 0.07
CHOL (mg/dL)	112.3 ± 0.01	119.01 ± 0.04	117.3 ± 0.02	113.4 ± 0.06	120.9 ± 0.03
Serum Protein (g/L)	6.65 ± 0.04	6.56 ± 0.02	6.44 ± 0.04	6.32 ± 0.09	6.61 ± 0.03
ALP (U/L)	239.9 ± 0.01	234.1 ± 0.09	228.4 ± 0.09	235.9 ± 0.07	237.9 ± 0.10
CRE (mg/dL)	0.51 ± 0.01	0.51 ± 0.01	0.50 ± 0.00	0.51 ± 0.01	0.51 ± 0.00
Blood Urea (mg/dL)	21.82 ± 0.10	23.18 ± 0.03	22.16 ± 0.04	21.82 ± 0.07	21.81 ± 0.06
Total Bilirubin (mg/dL)	0.51 ± 0.01	0.51 ± 0.02	0.50 ± 0.01	0.51 ± 0.00	0.51 ± 0.01
Bilirubin (direct) (mg/DL)	0.21 ± 0.01	0.22 ± 0.01	0.21 ± 0.00	0.22 ± 0.00	0.20 ± 0.02
Bilirubin (indirect)	0.27 ± 0.00	0.28 ± 0.01	0.26 ± 0.03	0.25 ± 0.02	0.28 ± 0.02
SGOT (U/L)	55.77 ± 0.14	61.07 ± 0.12	59.89 ± 0.13	62.91 ± 0.07	58.75 ± 0.11
SGPT (U/L)	41.15 ± 0.06	40.91 ± 0.09	41.51 ± 0.07	42.81 ± 0.05	41.97 ± 0.08
Globulin (gm%)	3.15 ± 0.04	3.35 ± 0.01	3.32 ± 0.02	3.13 ± 0.09	3.22 ± 0.05
A/G Ratio (Ratio)	0.82 ± 0.03	0.82 ± 0.05	0.81 ± 0.10	0.81 ± 0.03	0.82 ± 0.02

5.14.4 Gross pathology

When compared to the control group POL-6 treatment did not showed any difference in the relative organ weights (%) of both the sexes. The results of the relative organ weight of male and female rats are depicted in the Table 5.16.

Table 5.16: Relative organ weight (%) of rats treated orally with POL-6. Values expressed as mean ± SEM, n = 5 animals/group. $p<0.05$ (ANOVA/Dunnett's test) compared with respective control group. The relative organ weights of kidneys, testes, ovaries and adrenal glands mentioned are the combined weights of both the right and left organ.

Organ	Control	250 mg/kg	500 mg/kg	1000 mg/kg	High Dose Satellite
Male Rats					
Brain	1.91 ± 0.02	1.89 ± 0.03	1.90 ± 0.02	1.83 ± 0.02	1.91 ± 0.01
Heart	0.89 ± 0.00	0.92 ± 0.01	0.94 ± 0.00	0.98 ± 0.01	0.90 ± 0.01
Liver	6.92 ± 0.09	7.06 ± 0.10	6.91 ± 0.09	6.98 ± 0.14	7.59 ± 0.09
Spleen	0.62 ± 0.03	0.59 ± 0.03	0.60 ± 0.05	0.61 ± 0.06	0.58 ± 0.05
Thymus	0.39 ± 0.01	0.36 ± 0.01	0.34 ± 0.00	0.35 ± 0.03	0.38 ± 0.02
Kidney	2.92 ± 0.08	2.80 ± 0.07	2.71 ± 0.05	2.90 ± 0.05	2.82 ± 0.07
Adrenals	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.00
Testis	2.65 ± 0.01	2.91 ± 0.02	2.63 ± 0.03	2.81 ± 0.04	2.73 ± 0.02
Epididymis	0.84 ± 0.00	0.86 ± 0.02	0.81 ± 0.01	0.83 ± 0.01	0.84 ± 0.01
Female Rats					
Brain	1.87 ± 0.05	1.76 ± 0.08	1.84 ± 0.05	1.91 ± 0.07	1.87 ± 0.04
Heart	0.69 ± 0.01	0.73 ± 0.02	0.72 ± 0.01	0.68 ± 0.01	0.70 ± 0.03
Liver	6.23 ± 0.07	6.12 ± 0.56	6.48 ± 0.11	6.56 ± 0.09	6.42 ± 0.52
Spleen	0.52 ± 0.01	0.51 ± 0.02	0.49 ± 0.00	0.41 ± 0.01	0.53 ± 0.01
Thymus	0.34 ± 0.00	0.33 ± 0.01	0.36 ± 0.02	0.37 ± 0.01	0.36 ± 0.01
Kidney	1.85 ± 0.03	1.81 ± 0.03	1.82 ± 0.07	1.74 ± 0.06	1.86 ± 0.03
Adrenals	0.05 ± 0.00	0.06 ± 0.00	0.04 ± 0.00	0.05 ± 0.00	0.06 ± 0.00
Ovaries	0.13 ± 0.00	0.12 ± 0.00	0.11 ± 0.00	0.13 ± 0.00	0.11 ± 0.00
Uterus	0.52 ± 0.02	0.55 ± 0.03	0.54 ± 0.03	0.48 ± 0.05	0.49 ± 0.06

5.14.5 Histopathology

During histopathological examination POL-6 treated animals did not revealed any gross pathological and microscopic variations when compared with the normal control animals. Histopathological examination of the brain showed normal glial cells and normal myocardium morphology was seen in the heart tissues. POL-6 treatment did not induce any distinctive histological changes in brain and heart of the animals (Fig. 5.13).

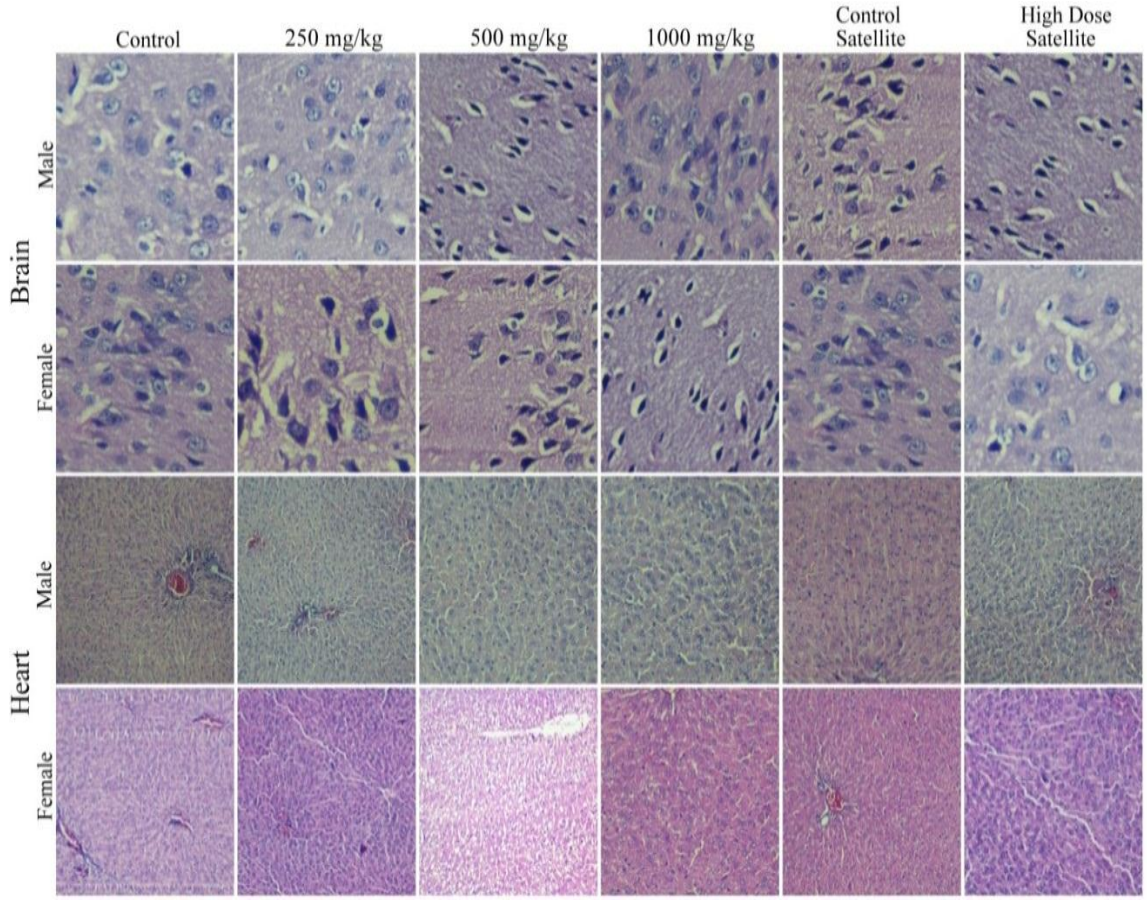


Fig 5.13: Histology of brain (H&E x100) and heart treated with POL-6 in the sub- acute toxicity study.

Microscopic examination of the liver showed normal hepatocytes with central and portal veins and normal nephrons without any interstitial bleeding, glomerular congestion and vacuolar degeneration of tubular cells were observed in the kidney of the rats. POL-6 treatment did not induce any distinctive histological changes in liver and kidney of the animals (Fig. 5.14).

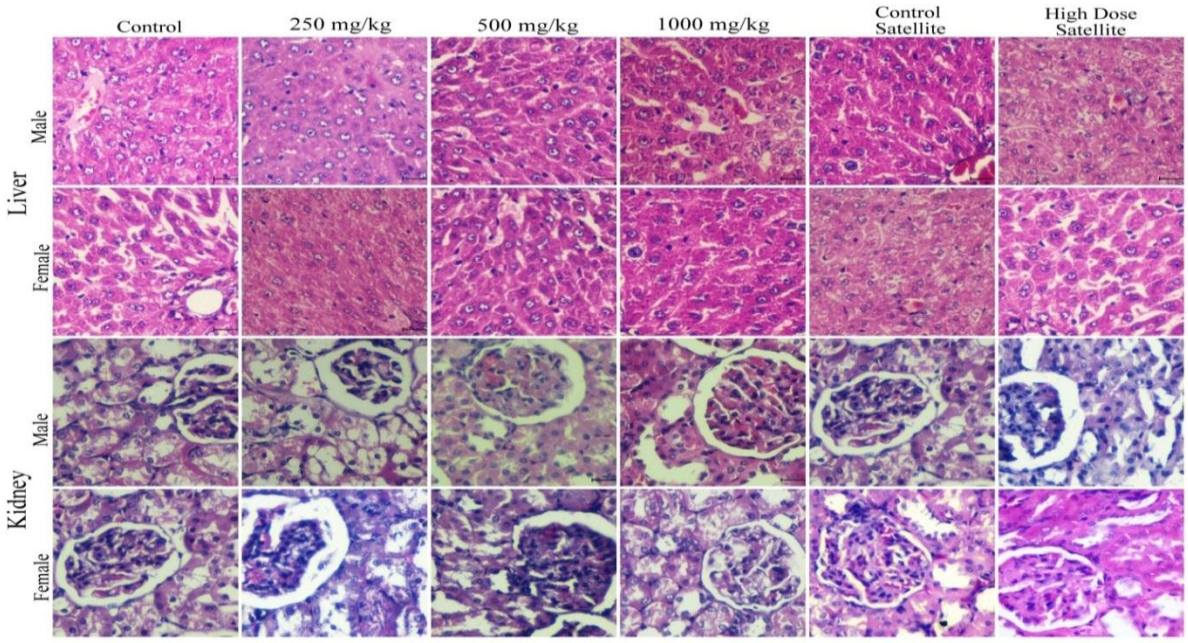


Fig 5.14: Histology of liver and kidney treated with POL-6 in the sub-acute toxicity study (H&Ex100).

Microscopic examination of the lungs showed normal air passages as well as normal pulmonary alveoli structure, no interstitial and basement membrane thickening and tearing was seen in the lungs treated with POL-6. Well conserved pancreatic exocrine lobules containing packed acinar units, which are sharp–delimitated from islets of Langerhans, were seen during the histological examination of pancreas. POL-6 treatment did not induce any characteristic histopathological changes in lungs and in pancreas of the rats (Fig. 5.15).

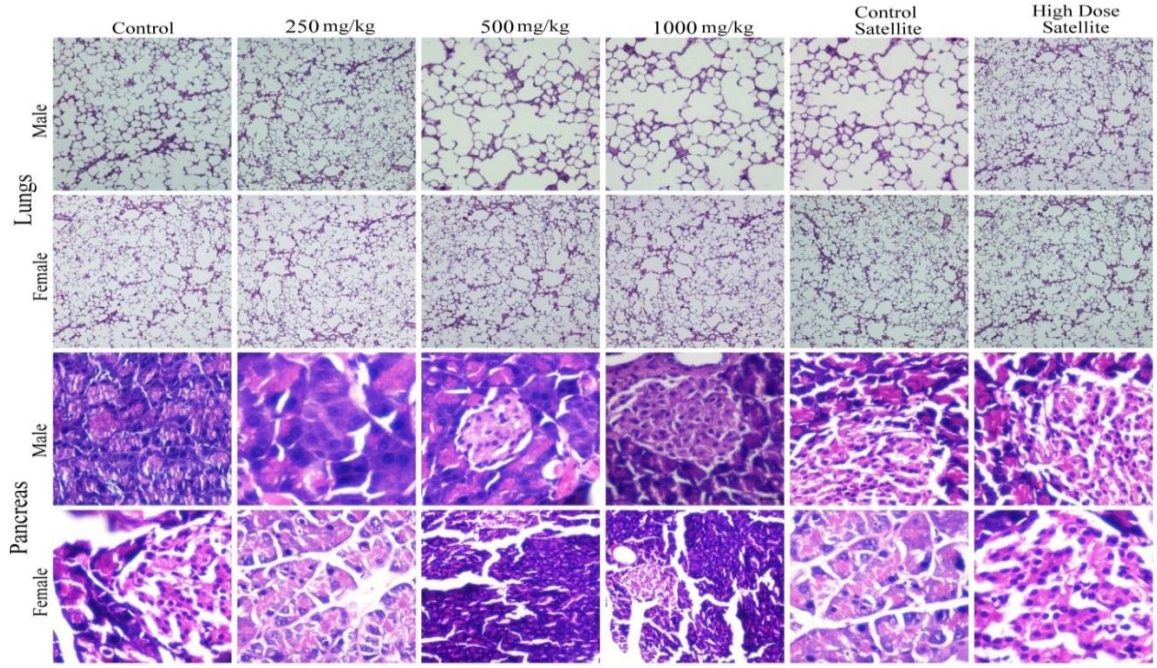


Fig 5.15: Histology of lungs and pancreas treated with POL-6 in the sub-acute toxicity study (H&Ex100).

Microscopic examination of spleen revealed normal mantle zones around lymphoid follicles, normal red and white pulp distinction and no hydropic degeneration and necrosis was there in the splenetic sections. Histology of sex organs revealed normal ovarian follicles in female rats and focal spermatogenesis in male rats. POL-6 treatment did not induce any characteristic histopathological changes in spleen and testis/ ovary of the rats (Fig. 5.16).

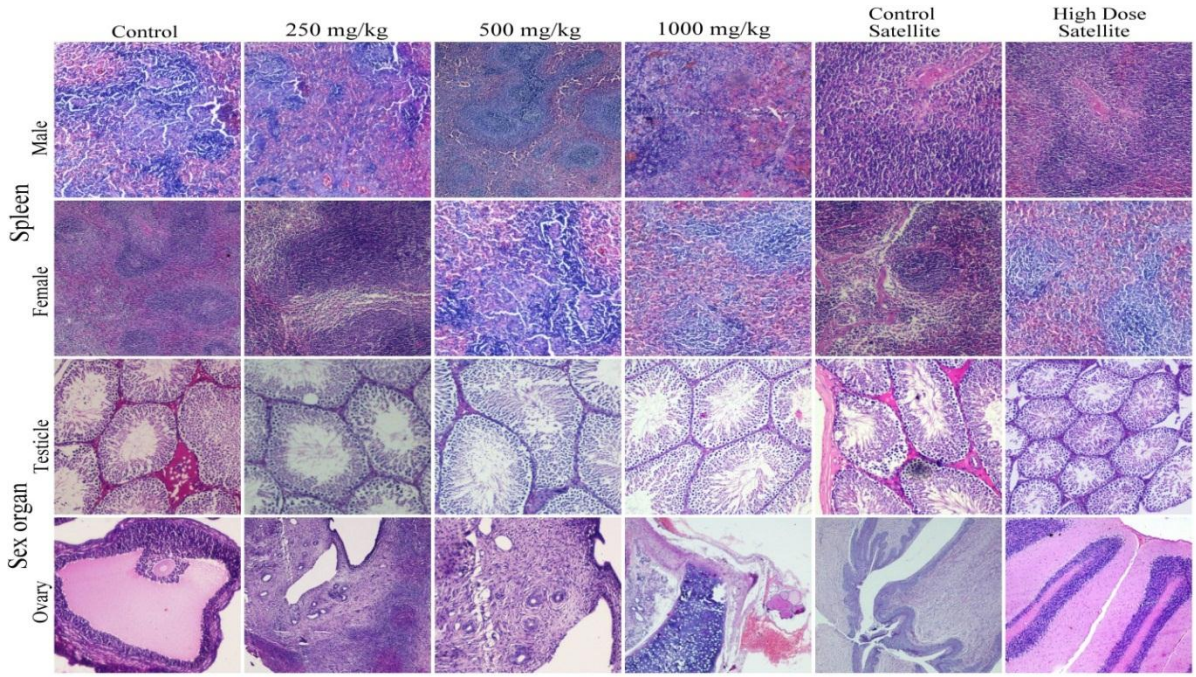


Fig 5.16: Histology of spleen and ovary/testis treated with POL-6 in the sub-acute toxicity study (H&E x100).

5.15 Ethanol withdrawal study

5.15.1 Ethanol consumption and body weight changes of the animals

Ethanol fed group animals were given free access of ethanol 4.5% v/v on the 1st day, 7.5% on the 2nd day and then maintained on 9% v/v ethanol from the 3rd day to 15th day. Ethanol intake by individual animal in ethanol fed groups was recorded daily for 15 days and calculated as g/kg/day. Daily ethanol consumption in ethanol fed groups varies between 13.57±1.85 to 17.12±1.34 g/kg during the exposure to 9% ethanol. No significant difference in ethanol intake was noticed among the ethanol fed groups. An increase in the body weight of approximately 11.2 % in control group animals and 6.4% in ethanol fed animals was observed over the initial body weight at the end of the study. Changes in animal's body weight observed during the study are depicted in Table 5.17.

Table 5.17: Body weight changes of the animals during acute toxicity study. Values are means ± SD; g = gram

Body Weight (g)			
Groups	**Beginning of the study**	**End of the study**	**Body weight change (%)**
Control	215.71 ± 4.56	239.86 ± 5.06	11.2%
Alcohol fed	213.30 ± 5.49	226.95 ± 6.01	6.4%

5.15.2 Blood alcohol concentration measurement

Blood alcohol concentration (BAC) was measured on day 15th and on alcohol withdrawal days 16th, 17th and 18th and was expressed as mean ± SEM. On day 15th of the study, BAC was found to be 111.6 ± 8.151 mg %. Very low concentration of alcohol was observed in the animals after 24 hrs of alcohol withdrawal (8.012 ± 1.325 mg %), 2.017 ±0.285 mg % after 48 hrs of alcohol withdrawal, 0.00 ± 0.00 mg % after 72 hrs of alcohol withdrawal.

5.15.3 Behavioral Parameters Study

5.15.3.1 Anxiety tests

5.15.3.1.1 Elevated plus maze

When tested on the EPM ethanol fed animals revealed a significant decrease ($p<0.001$) in the time spend and in number of entries into the open arms when compared to the normal control group. A significant increase ($p<0.001$) in the time spent and in number of entries into the closed arms was also observed in the ethanol fed animals when compared to the normal group animals (Fig. 5.17 A, B, C and D). These findings revealed the development of anxiety in the ethanol withdrawal animals. Treatment with POL-6 (20, 50 and 100mg/kg, oral) and diazepam (2mg/kg) for three consecutive days produced a significant ($p<0.001$) increase in the time spend and in number of entries into the open arms and significant decrease ($p<0.001$) in the time spend and in number of entries into the closed arms when compared to the disease control rats (Fig. 5.17 A, B, C and D).

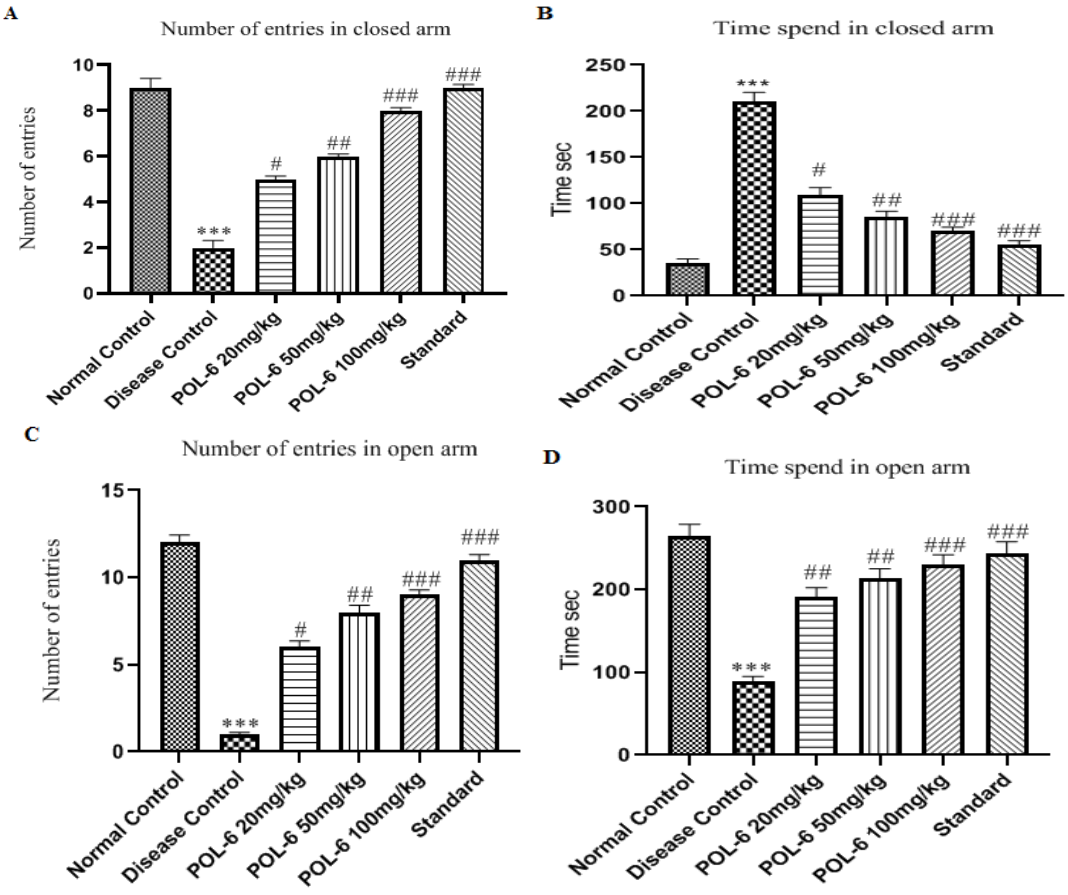


Fig 5.17: Effect of drug treatment on ethanol withdrawal anxiety when tested on elevated plus maze in rats. (Fig. A) Number of entries in the closed arm. (Fig. B) Time spent in the closed arm. (Fig. C) Number of entries in the open arm. (Fig. D) Time spent in the open arm. *p<0.05, **p<0.01, ***p<0.001 (Compared to normal control); #p<0.05, ##p<0.01, ###p<0.001 (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test.

5.15.3.1.2 Light and dark model

When tested on the light and dark model ethanol fed animals revealed a significant decrease ($p<0.001$) in the time spent and in number of entries into the light chamber of the light and dark model when compared to the normal control animals. A significant increase ($p<0.001$) in the time spend and in number of entries into the dark chamber of light and dark model was also noticed in the ethanol fed animals when compared to the normal control animals (Fig. 5.18 A, B, C D). Similar to the EPM, the results from the light and dark model revealed the development of anxiety in the ethanol withdrawal animals. Treatment with POL-6 (20, 50 and 100mg/kg, oral) and diazepam (2mg/kg) for three consecutive days produced a significant ($p<0.001$) increase in the time spend and in number of entries into the light chamber and significant decrease ($p<0.001$) in the time spend and in number of entries into the dark chamber when compared to the disease control rats (Fig. 5.18 A, B, C D).

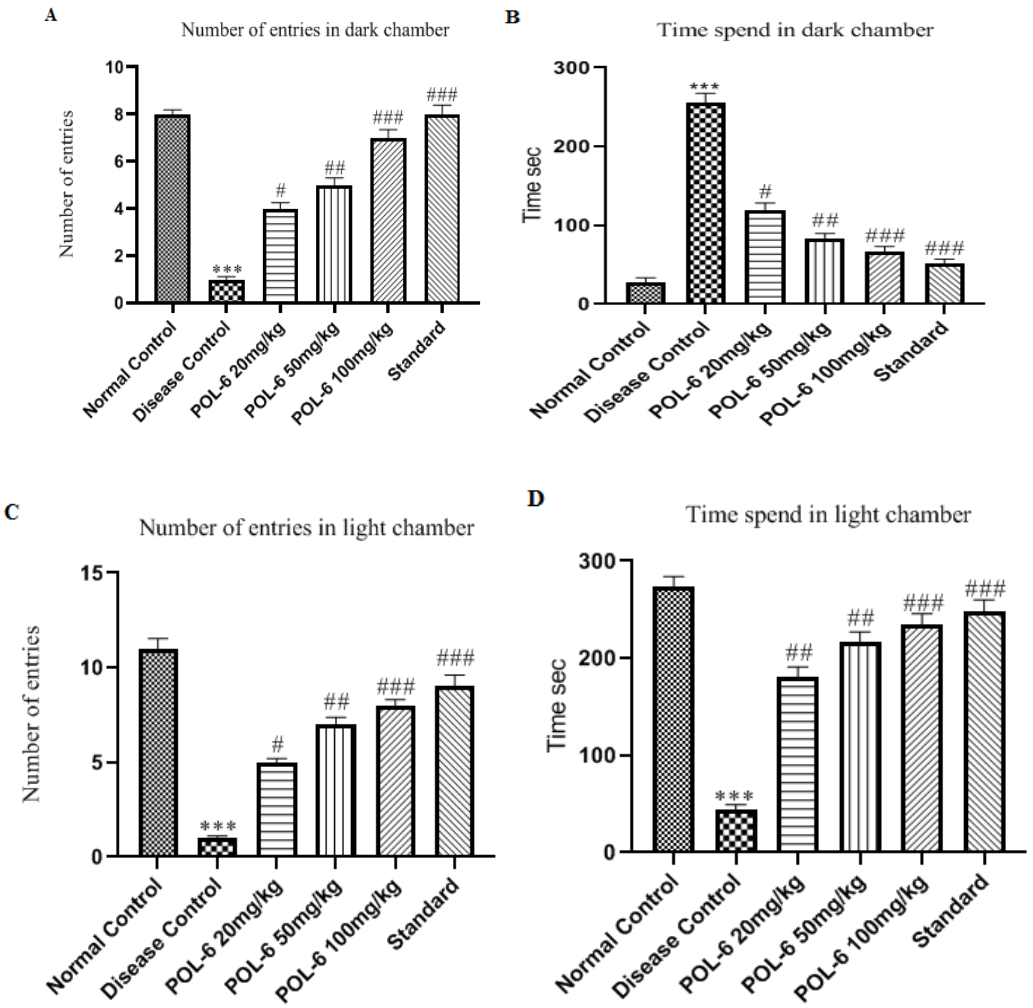


Fig 5.18: Effect of drug treatment on ethanol withdrawal anxiety when tested on light and dark model in rats. (Fig. A) Number of entries in the dark chamber (Fig. B) Time spend in the dark chamber.(Fig. C) Number of entries in the light chamber (Fig. D) Time spend in the light chamber. *p<0.05, **p<0.01, ***p<0.001 (Compared to normal control); #p<0.05, ##p<0.01, ###p<0.001 (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test.

5.15.3.2 Depression tests

Rats were subjected to the depression paradigm on the 3rd day of alcohol withdrawal (18th day of the study). Fluoxetine (5 mg/kg i.p) was used as a standard drug during depression paradigm study.

5.15.3.2.1 Forced swim test

Ethanol-fed rats revealed a significant increase ($p<0.001$) in the immobility time when compared to the immobility time of the normal group rats in the FST (Fig 5.19). The results from the FST indicate the development of depression in the ethanol withdrawal animals. Treatment with POL-6 (20, 50 and 100mg/kg, oral) and fluoxetine (5 mg/kg i.p) for three

consecutive days produced the significant ($p<0.001$) decrease in the immobility time when compared to the immobility time of the disease control animals in the FST (Fig 5.19).

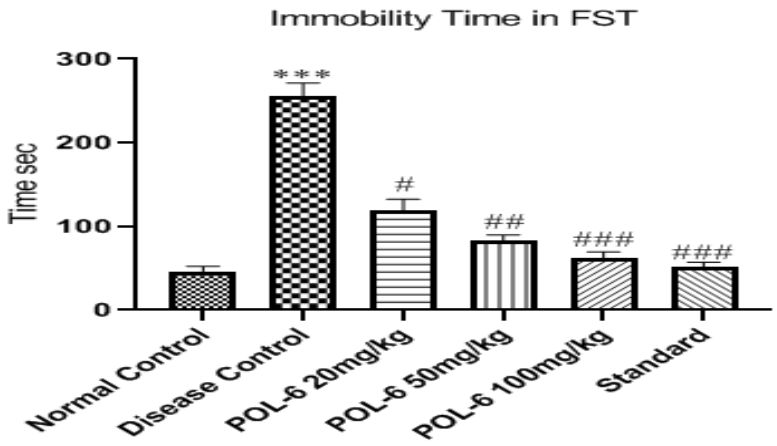


Fig 5.19: Effect of drug treatment on ethanol withdrawal depressive like behavior when tested in forced swim test in rats. *p<0.05, **p<0.01, ***p<0.001 (Compared to normal control); #p<0.05, ##p<0.01, ###p<0.001 (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test.

5.15.3.2.2 Tail suspension test

In TST, ethanol-fed rats revealed a significant increase ($p<0.001$) in the immobility time when compared to the immobility time of the normal group animals (Fig 5.20). Similar to the FST, the results from the TST indicate the development of depression in the ethanol withdrawal animals. Treatment with POL-6 (20, 50 and 100mg/kg, oral) and fluoxetine (5 mg/kg i.p) for three consecutive days produced the significant ($p<0.001$) decrease in the immobility time when compared to the immobility time of the disease control animals in the TST (Fig 5.20).

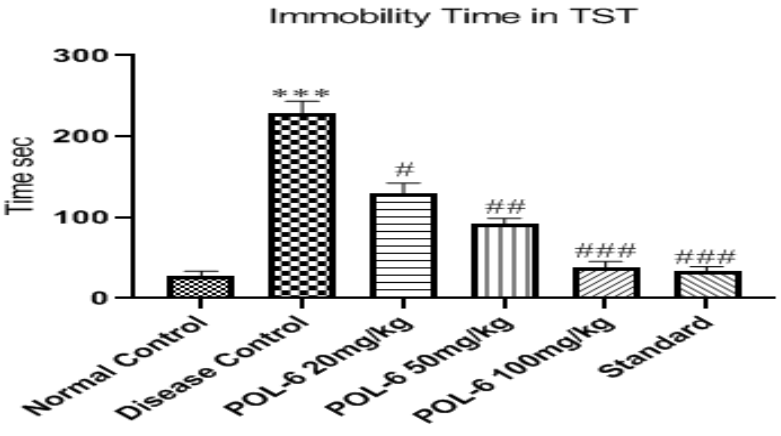


Fig 5.20: Effect of drug treatment on ethanol withdrawal depressive like behavior when tested in forced swim test in rats. *p<0.05, **p<0.01, ***p<0.001 (Compared to normal control); #p<0.05, ##p<0.01, ###p<0.001 (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test.

5.15.3.3 Seizures test

Effect of POL-6 was tested on the alcohol withdrawal seizures in the animals on the 3rd day of alcohol withdrawal (18th day of the study). For studying alcohol withdrawal seizures a sub convulsive dose of the Pentylenetetrazol (30mg/kg i.p.) was administered to the alcohol fed animals and seizure scoring was recorded thereafter.

5.15.3.3.1 Seizure scoring

Pentylenetetrazol administration at sub convulsive dose (30mg/kg i.p.) causes an increase in the seizure severity and frequency in the ethanol withdrawal rats. No seizures were observed in the normal control animals after Pentylenetetrazol administration at sub convulsive dose (30mg/kg i.p.). Treatment with POL-6 (20, 50, 100 mg/kg, oral) and Diazepam (2 mg/kg) showed decreased seizure severity and frequency in the alcohol fed animals. The seizures were recorded as; Score 0: No reaction, Score 1: Ear and facial jerking Score 2: convulsive wave all through the body, Score 3: Myoclonic jerks, Score 4: Clonic tonic spasms, transform over into side position, Score 5: Generalized clonic tonic seizures with loss of postural control, Score 6: Death. The results of the seizure scoring are showed in the Table 5.18.

Table 5.18: Seizure severity in the animals.

GROUP 1	**Score 0**	**Score 1**	**Score 2**	**Score 3**	**Score 4**	**Score 5**	**Score 6**
No. of animals with score	6	0	0	0	0	0	0
GROUP 2	**Score 0**	**Score 1**	**Score 2**	**Score 3**	**Score 4**	**Score 5**	**Score 6**
No. of animals with score	0	6	6	6	6	6	3
GROUP 3	**Score 0**	**Score 1**	**Score 2**	**Score 3**	**Score 4**	**Score 5**	**Score 6**
No. of animals with score	0	3	6	3	0	0	0
GROUP 4	**Score 0**	**Score 1**	**Score 2**	**Score 3**	**Score 4**	**Score 5**	**Score 6**
No. of animals with score	0	5	6	3	0	0	0
GROUP 5	**Score 0**	**Score 1**	**Score 2**	**Score 3**	**Score 4**	**Score 5**	**Score 6**
No. of animals with score	0	6	6	2	0	0	0
GROUP 6	**Score 0**	**Score 1**	**Score 2**	**Score 3**	**Score 4**	**Score 5**	**Score 6**
No. of animals with score	0	4	6	2	0	0	0

5.15.3.4 Locomotor activity test

5.15.3.4.1 Actophotometer

When animals were tested on the actophotometer during the alcohol withdrawal period the ethanol-fed rats showed a significant ($p<0.001$) locomotor hyperactivity when compared with the normal group animals (Fig. 5.21). The results from the actophotometer indicate the development of locomotor hyperactivity in the ethanol withdrawal animals. Treatment with POL-6 (20, 50 and 100mg/kg, oral) and diazepam (2mg/kg) for three consecutive days significantly ($p<0.001$) reduced the ethanol withdrawal locomotor hyperactivity when compared to the disease control animals.

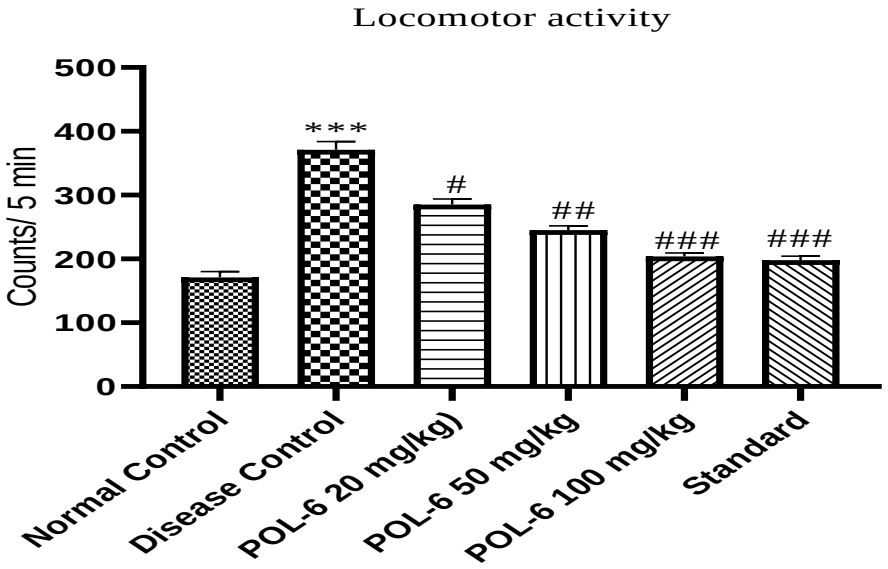


Fig 5.21: Effect of drug treatment on alcohol withdrawal locomotor hyperactivity when tested on Actophotometer. *p<0.05, **p<0.01, ***p<0.001 (Compared to normal control); #p<0.05, ##p<0.01, ###p<0.001 (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test.

5.15.4 Hematological and biochemical parameters evaluation

5.15.4.1 Hematological tests

Alcohol treatment for 15 days and POL-6 (20, 50, 100mg/kg, oral) and diazepam (2mg/kg) treatment for following 3 days in alcohol fed rats did not induce any significant changes in the hematological parameters when compared with the normal group rats. The results of hematological examination are depicted in the Table 5.19.

Table 5.19: Effect of drug treatment on Hematological parameters. Values expressed as mean ± SEM, n = 5 animals/group. $p<0.05$ (ANOVA/Dunnett's test) compared with respective control group.

Parameter	Normal Control	Disease Control	POL-6 20 mg/kg	POL-6 50 mg/kg	POL-6 100 mg/kg	Diazepam
WBC(10^3µL)	5.16 ± 1.21	5.06 ± 1.31	4.91 ± 0.99	4.99 ± 1.13	5.09 ± 1.42	4.72 ± 0.89
LYM -R%	76.62 ± 2.03	77.93 ± 1.91	75.03 ± 1.45	79.03 ± 1.31	78.51 ± 2.21	78.43 ± 0.93
MON-R %	1.27 ± 0.21	1.23 ± 0.36	1.19 ± 0.19	1.16 ± 0.21	1.21 ± 0.17	1.22 ± 0.13
NEU-R %	20.14 ± 1.72	18.56 ± 1.31	17.97 ± 0.93	18.89 ± 1.02	21.47 ± 1.21	19.61 ± 1.04
EOS-R %	1.05 ± 0.15	1.04 ± 0.14	1.02 ± 0.18	1.06 ± 0.13	1.04 ± 0.20	1.03 ± 0.19
RBC (10^6/µL)	5.41 ± 0.16	5.71 ± 0.36	5.72 ± 0.32	5.08 ± 0.18	5.31 ± 0.17	5.19 ± 0.21
HGB (g/dL)	13.59 ± 0.74	13.12 ± 0.44	13.33 ± 0.17	13.45 ± 0.34	13.29 ± 0.18	13.39 ± 0.25
MCV (µm^3)	89.25 ± 1.03	87.98 ± 1.42	88.11 ± 0.98	86.77 ± 1.62	87.12 ± 0.95	89.09 ± 0.91
MCH (pg)	25.98 ± 1.01	23.99 ± 0.93	24.19 ± 1.04	25.13 ± 0.98	24.23 ± 1.17	25.02 ± 0.99
PLT (10^3/µL)	2.94 ± 0.09	2.54 ± 0.06	2.47 ± 0.09	2.49 ± 0.07	2.56 ± 0.08	2.48 ± 0.10
ESR (mm/1^{st}hr)	1.53 ± 0.31	1.49 ± 0.28	1.48 ± 0.21	1.55 ± 0.30	1.49 ± 0.31	1.57 ± 0.25
PCV (%)	43.08 ± 1.09	42.98 ± 0.97	42.72 ± 0.94	41.99 ± 0.74	43.09 ± 0.76	42.09 ± 0.81

5.15.4.2 Biochemical parameters evaluation

5.15.4.2.1 Liver function tests

The effect of POL-6 was explored on the traditional alcohol liver markers like GGT, SGOT and SGPT. Alcohol administration in alcohol fed animals for following 15 days significantly ($p<0.001$) elevated the levels of SGOT, SGPT and GGT when compared to the normal control animals. POL-6 (20, 50, 100 mg/kg, oral) and diazepam (2mg/kg) treatment for following 3 days significantly ($p<0.001$) reversed the elevated levels of GGT, SGOT and SGPT when compared to the disease control animals. However alcohol, POL-6 and diazepam treatment did not bring about any significant change in the total bilirubin, albumin and globulin levels when compared to the normal group animals. The results of alcohol liver markers are shown in the Table 5.20.

Table 5.20: Effect of drug treatment on liver markers. [a]p<0.001 (Compared to normal control); [b]p<0.001 (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test.

Parameters	Normal Control	Disease Control	POL-6 (20 mg/kg)	POL-6 (50 mg/kg)	POL-6 (100 mg/kg)	Diazepam (2 mg/kg)
Total Bilirubin (mg/dl) (0.1-0.55)	0.50±0.02	0.51±0.04	0.49±0.10	0.47±0.08	0.42±0.19	0.51±0.06
SGOT(µ/l) (20-70)	54.02±10.66	109±11.71[a]	83.5±9.42	73.1±16.14[b]	57±13.48[b]	53±8.80[b]
SGPT (µ/l) (10-30)	26.33±7.44	60.5±9.83[a]	48.2±6.91[b]	40.8±7.92[b]	33.23±10.4[b]	34.34±7.2[b]
Albumin gm(%) (2.8-4.8)	4.5±0.14	4.2±0.23	4.2±1.42	4.3±1.01	4.4±0.18	4.5±0.46
Globulin gm(%) (1.8-3.2)	3.1±0.04	3.2±0.18	3.1±0.41	3.0±1.63	3.1±1.68	3.2±0.41
GGT(iu/l) (10-70)	18.25±1.12	78.9±1.8[a]	67.10±1.79	51.7±1.43[b]	29.4±0.79[b]	26.8±0.42[b]

5.15.4.2.2 Kidney function tests

Alcohol treatment for 15 days and POL-6 (20, 50, 100mg/kg, oral) and diazepam (2mg/kg) treatment in alcohol fed rats for following 3 days did not produce any significant change in the kidney function tests like blood urea, serum creatinine and serum uric acid when compared to the normal group animals. The results of the kidney function tests are depicted in the Table 5.21.

Table 5.21: Effect of drug treatment on Kidney function. #p<0.001 (Compared to normal control); *****p<0.001 (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test.

Groups	Blood urea (mg/dl)(10-32)	Serum creatinine (mg/dl)(0.2-0.8)	Serum uric acid (mg/dl)(3-6)
Normal Control	28.08±2.55	0.80± 0.13	3.66±0.19
Disease Control	27.98±3.81	0.81±0.03	3.86±0.36
POL-6 (20 mg/kg)	29.48± 2.51	0.82±0.07	4.12±0.27
POL-6 (50 mg/kg)	26.03± 2.81	0.80±0.04	3.91±0.31
POL-6 (100 mg/kg)	29.13± 1.47	0.80±0.02	3.08±0.44
Diazepam (2mg/kg)	27.00± 2.20	0.81± 0.06	3.23±0.54

5.15.5 RT-PCR analysis

Effect of drug treatment on mRNA expression in $GABA_A$ receptor and NMDA-glutamate receptor subunits in Pre-frontal Cortex, Amygdala and Hippocampus was analyzed by RT-PCR (Appendix B).

5.15.5.1 Prefrontal cortex

5.15.5.1.1 $GABA_A$ subunits

Fig. 5.22 A, B, C, D and E demonstrates the influences of POL-6 on the altered expression of $GABA_A$ receptor subunits in the rats' prefrontal cortex. Two-way ANOVA represented the influences of POL-6 on the relative expression of Gabra (1-5) in the prefrontal cortex. Dunnett's multiple comparison test revealed that the relative gene expression of Gabra1, Gabra2, Gabra3, Gabra4 and Gabra 5 were significantly ($p<0.001$) down regulated in the disease control rats when compared with the normal control rats. Interestingly POL-6 (20, 50 and 100 mg/kg, oral) treatment for following 3 days significantly ($p<0.001$) normalized the down regulated genes Gabra1, Gabra 2, Gabra 3 and Gabra 5 when compared to the disease control rats. No significant changes were detected in the expression of Gabra 4 on the treatment with POL-6 (20, 50 and 100 mg/kg, oral). Diazepam (2mg/kg) treatment significantly ($p<0.001$) normalized the down regulated genes Gabra1, Gabra 2, Gabra4 and Gabra 5 as compared to the disease control rats however; no significant changes were detected in the expression of Gabra 3 on the treatment with Diazepam (2mg/kg).

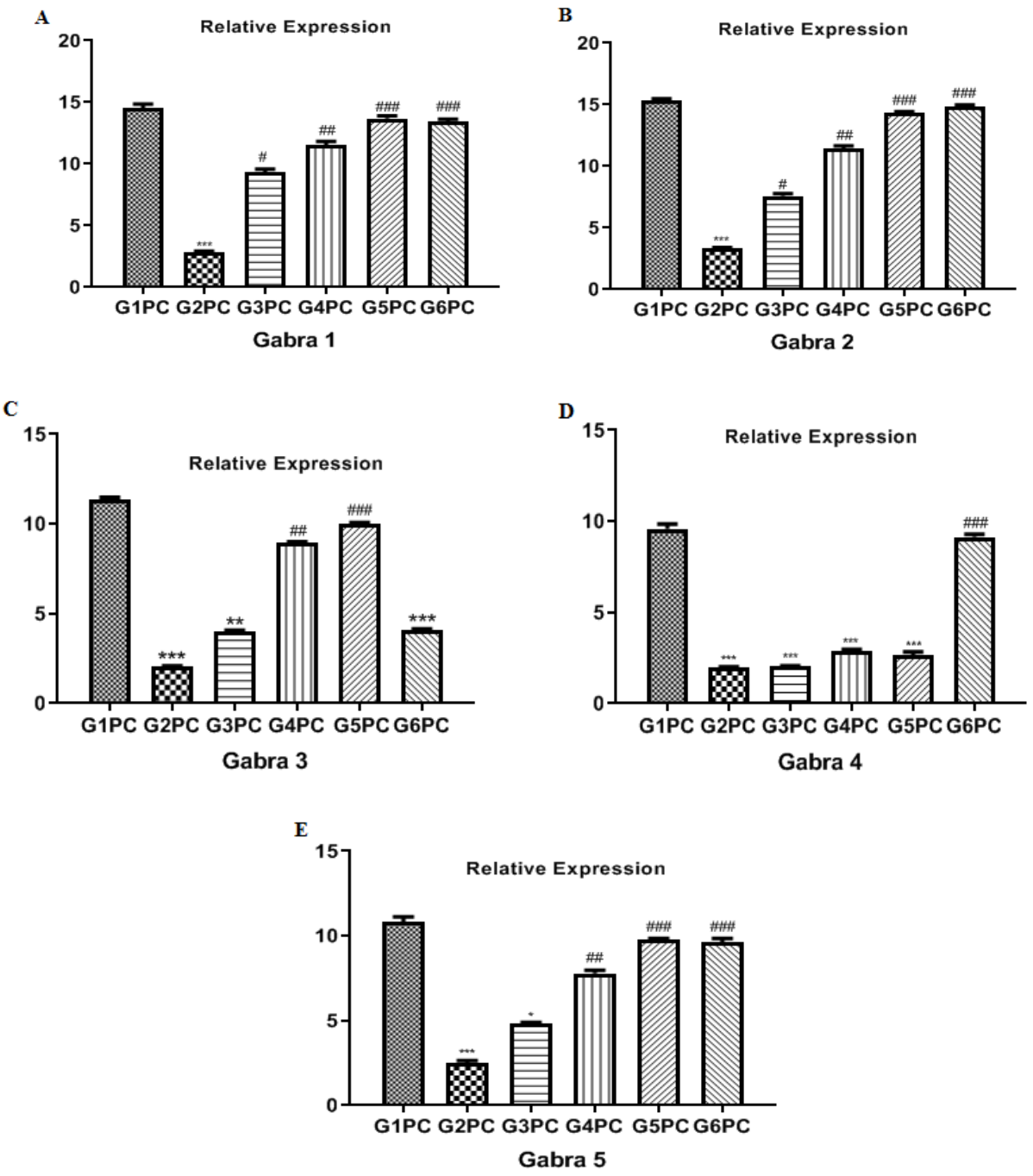


Fig 5.22: Effect of drug treatment on the relative mRNA expression of $GABA_A$ subunits in prefrontal cortex (PC) of the rats. (Fig. A) Gabra 1 (Fig. B) Gabra 2 (Fig. C) Gabra 3 (Fig. D) Gabra 4 (Fig. E) Gabra 5**.** *p<0.05, **p<0.01, ***p<0.001 (Compared to normal control); #p<0.05, ##p<0.01, ###p<0.001 (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test. G1 = Normal control, G2 = Disease control, G3 = Polyherbal 20mg/kg, G4 = Polyherbal 50mg/kg, G5 = Polyherbal 100mg/kg, G6 = Diazepam 2mg/kg.

5.15.5.1.2 NMDA subunits

Fig. 5.23 A, B and C demonstrates the influences of POL-6 on the altered expression of NMDA subunits in the rats' prefrontal cortex. Two-way ANOVA represented the influences

of POL-6 on the relative expression of Grin1, Grin2a and Grin2b in the prefrontal cortex. Dunnett's multiple comparison test revealed that the relative expression of Grin1, Grin2a and Grin2b were significantly ($p<0.001$) up regulated in the disease control rats when compared to the normal control rats. Interestingly POL-6 (20, 50 and 100 mg/kg, oral) treatment for following 3 days significantly ($p<0.001$) normalized the up regulated genes Grin1 and Grin2a when compared to the disease control rats. No significant changes were detected in the gene expression of Grin2b on the treatment with POL-6 (20, 50 and 100 mg/kg, oral). Diazepam (2mg/kg) treatment significantly ($p<0.001$) reversed the down regulated genes Grin1, Grin2a and Grin2b in comparison to the disease control rats.

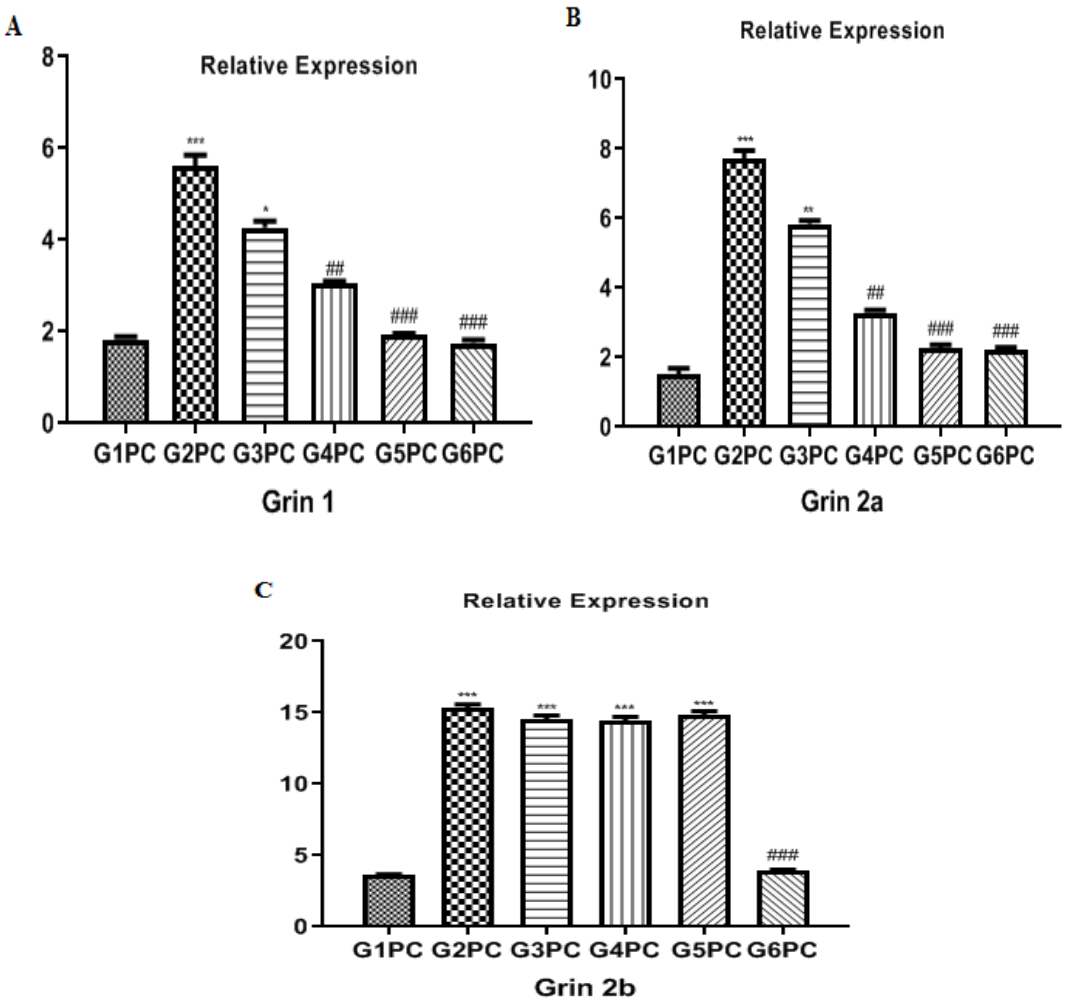


Fig 5.23: Effect of drug treatment on the relative mRNA expression of NMDA subunits in prefrontal cortex (PC) of the rats. (Fig. A) Grin 1 (Fig. B) Grin 2a (Fig. C) Grin 2b *p<0.05, **p<0.01, ***p<0.001 (Compared to normal control); #p<0.05, ##p<0.01, ###p<0.001 (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test. G1 = Normal control, G2 = Disease control, G3 = Polyherbal 20mg/kg, G4 = Polyherbal 50mg/kg, G5 = Polyherbal 100mg/kg, G6 = Diazepam 2mg/kg.

5.15.5.2 Amygdala

5.15.5.2.1 $GABA_A$ subunits

Fig. 5.24 A, B, C, D, and E demonstrates the influences of POL-6 on the altered expression of $GABA_A$ receptor subunits in rats' amygdala. Two-way ANOVA represented the influences of POL-6 on the relative expression of Gabra1, Gabra2, Gabra3, Gabra4 and Gabra 5 in the amygdala. Dunnett's multiple comparison tests showed that the relative expression of Gabra1, Gabra2, Gabra3, Gabra4 and Gabra 5 were significantly ($p<0.001$) down regulated in the disease control rats when compared to the normal control rats.

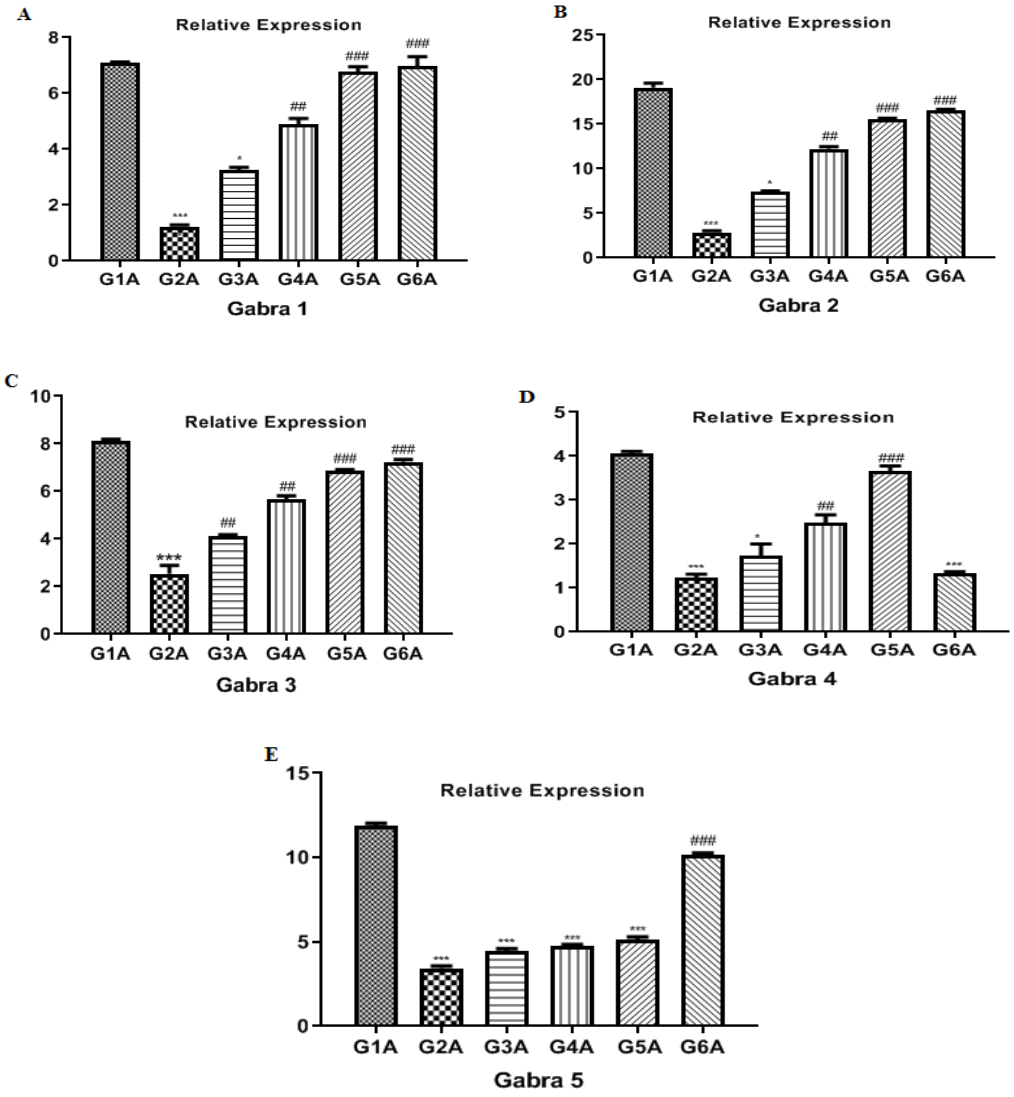


Fig 5.24: Effect of drug treatment on the relative mRNA expression of $GABA_A$ subunits in amygdala (A) of the rats. (Fig. A) Gabra 1 (Fig. B) Gabra 2 (Fig. C) Gabra 3 (Fig. D) Gabra 4 (Fig. E) Gabra 5**.** *p<0.05, **p<0.01, ***p<0.001 (Compared to normal control); #p<0.05, ##p<0.01, ###p<0.001 (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test. G1 = Normal control, G2 = Disease control, G3 = Polyherbal 20mg/kg, G4 = Polyherbal 50mg/kg, G5 = Polyherbal 100mg/kg, G6 = Diazepam 2mg/kg.

Interestingly POL-6 (20, 50 and 100 mg/kg, oral) treatment for following 3 days significantly ($p<0.001$) normalized the down regulated genes Gabra1, Gabra 2, Gabra 3 and Gabra 4 in comparison to the disease control rats. No significant changes were detected in the gene expression of Gabra 5 on the treatment with POL-6 (20, 50 and 100 mg/kg, oral). Diazepam (2mg/kg) treatment significantly ($p<0.001$) normalized the down regulated genes Gabra1, Gabra 2, Gabra3 and Gabra 5 in comparison to the disease control rats however; no significant changes were detected in the gene expression of Gabra 4 on the treatment with Diazepam (2mg/kg).

5.15.5.2.2 NMDA subunits

Fig. 5.25 A, B and C demonstrates the influences of POL-6 on the altered expression of NMDA subunits in rats' amygdala.

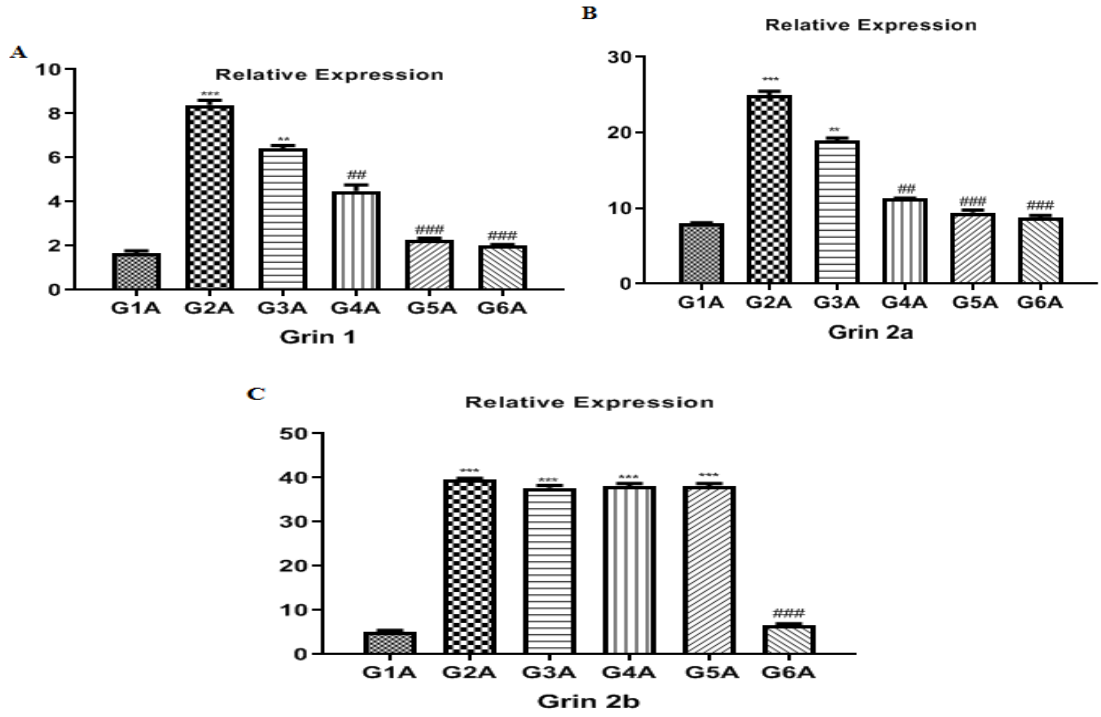


Fig 5.25: Effect of drug treatment on the relative mRNA expression of NMDA subunits in amygdala (A) of the rats. (Fig. A) Grin 1 (Fig. B) Grin 2a (Fig. C) Grin 2b *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Compared to normal control); #$p<0.05$, ##$p<0.01$, ###$p<0.001$ (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test. G1 = Normal control, G2 = Disease control, G3 = Polyherbal 20mg/kg, G4 = Polyherbal 50mg/kg, G5 = Polyherbal 100mg/kg, G6 = Diazepam 2mg/kg.

Two-way ANOVA represented the influences of POL-6 on the relative expression of Grin1, Grin2a and Grin2b in the amygdala. Dunnett's multiple comparison tests showed that the relative expression of Grin1, Grin2a and Grin2b were significantly ($p<0.001$) up regulated in

the disease control rats when compared to the normal control rats. Interestingly POL-6 (20, 50 and 100 mg/kg, oral) treatment for following 3 days significantly ($p<0.001$) normalized the up regulated genes Grin1 and Grin2a in comparison to the disease control rats. No significant changes were detected in the gene expression of Grin2b on the treatment with POL-6 (20, 50 and 100 mg/kg, oral). However, Diazepam (2mg/kg) treatment significantly ($p<0.001$) reversed the down regulated genes Grin1, Grin2a and Grin2b in comparison to the disease control rats.

5.15.5.3 Hippocampus

5.15.5.3.1 $GABA_A$ subunits

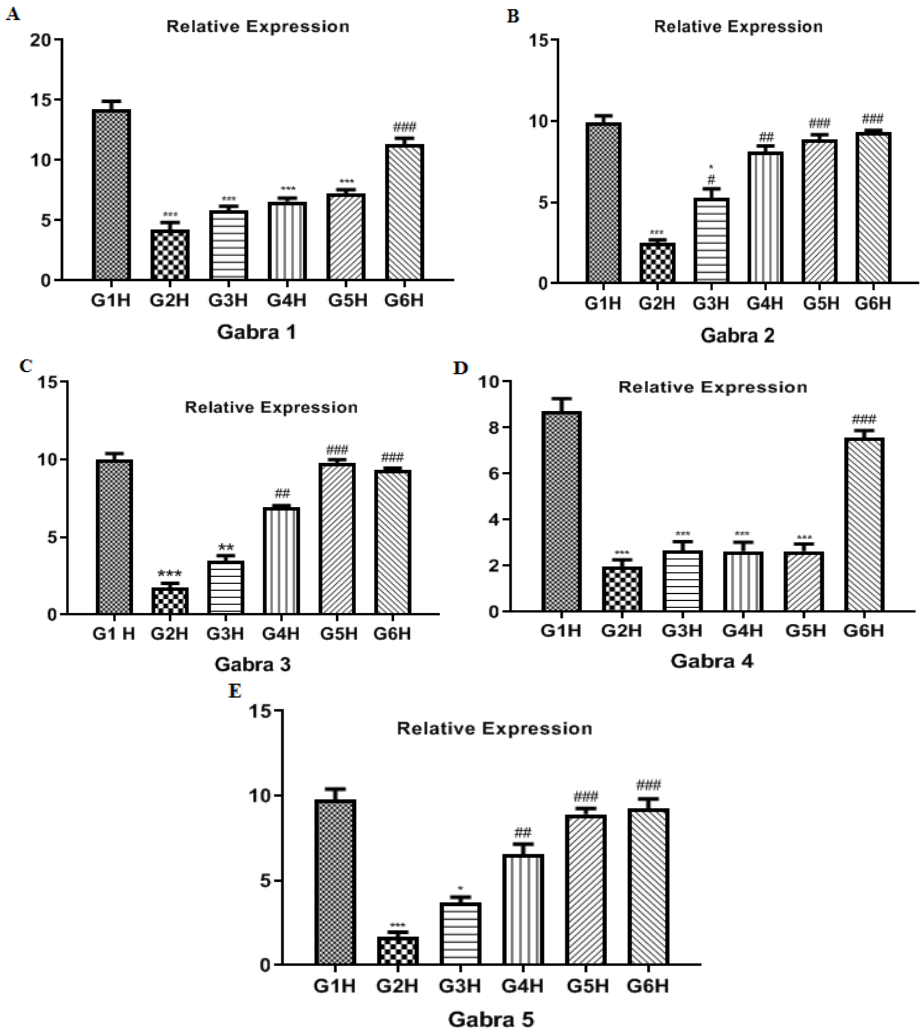


Fig 5.26: Effect of drug treatment on the relative mRNA expression of $GABA_A$ subunits in hippocampus (H) of the rats. (Fig. A) Gabra 1 (Fig. B) Gabra 2 (Fig. C) Gabra 3 (Fig. D) Gabra 4 (Fig. E) Gabra 5**.** *p<0.05, **p<0.01, ***p<0.001 (Compared to normal control); #p<0.05, ##p<0.01, ###p<0.001 (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test. G1 = Normal control, G2 = Disease control, G3 = Polyherbal 20mg/kg, G4 = Polyherbal 50mg/kg, G5 = Polyherbal 100mg/kg, G6 = Diazepam 2mg/kg.

5.15.5.3.2 NMDA subunits

Fig. 5.27 A, B and C demonstrates the influences of POL-6 on the altered expression of NMDA subunits in the rat's hippocampus. Two-way ANOVA represented the influences of POL-6 on the relative expression of Grin1, Grin2a and Grin2b in the hippocampus. Dunnett's multiple comparison tests showed that the relative expression of Grin1, Grin2a and Grin2b were significantly ($p<0.001$) up regulated in the disease control rats when compared to the normal control rats. Interestingly POL-6 (20, 50 and 100 mg/kg, oral) treatment for following 3 days significantly ($p<0.001$) normalized the up regulated gene Grin1 in comparison to the disease control rats. No significant changes were detected in the expression of Grin 2a and Grin2b on the treatment with POL-6 (20, 50 and 100 mg/kg, oral). However, Diazepam (2mg/kg) treatment significantly ($p<0.001$) reversed the down regulated genes Grin1, Grin2a and Grin2b in comparison to the disease control rats.

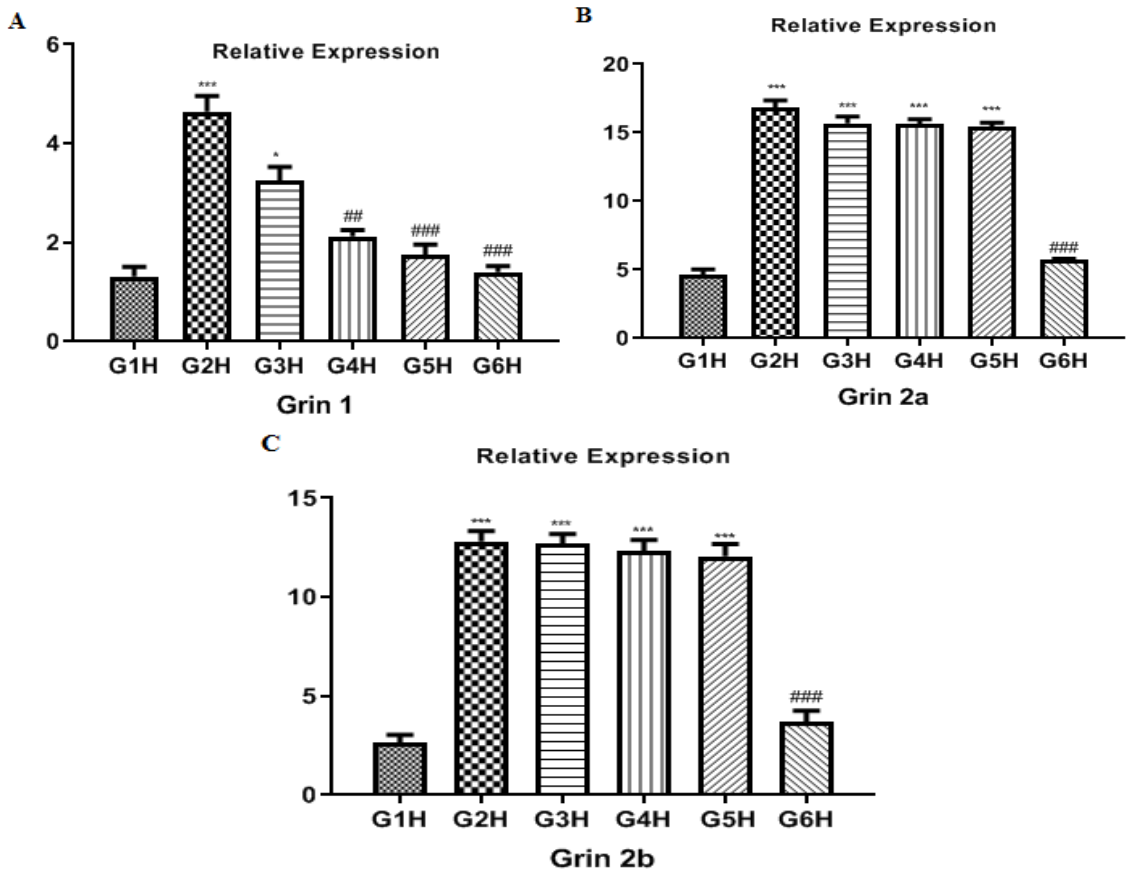


Fig 5.27: Effect of drug treatment on the relative mRNA expression of NMDA subunits in hippocampus (H) of the rats. (Fig. A) Grin 1 (Fig. B) Grin 2a (Fig. C) Grin 2b *p<0.05, **p< 0.01, ***p<0.001 (Compared to normal control); #p<0.05, ##p<0.01, ###p<0.001 (Compared to disease control); One-way ANOVA; Dunnett's multiple comparison test. G1 = Normal control, G2 = Disease control, G3 = Polyherbal 20mg/kg, G4 = Polyherbal 50mg/kg, G5 = Polyherbal 100mg/kg, G6 = Diazepam 2mg/kg.

CHAPTER 6

DISCUSSION

Plants are the basis of both traditional medicines and modern drug discoveries. The global interest is on herbal medicines due to low better tolerance, less expensive and minimum side effects. More than 50,000 plant species are used for therapeutic purposes [245-247]. In Ayurveda, an Indian system of medicine formulation is classified on the basis of two principles: single drug usage or using multiple drugs known as polyherbal formulation or herb-herb combination [248]. Due to synergism, polyherbal preparations express high effectiveness which is not accessible in single herbal formulations. The synergism in drug combination therapy acts in pharmacokinetic and pharmacodynamics manner, thus targeting disease with diverse mechanism of action [248]. In the present study POL-6 preparation containing 6 plants extracts *Bacopa monnieri, Hypericum perforatum, Centella asiatica, Withania somnifera, Camellia sinesis and Ocimum sanctum* was prepared, standardized and explored for its pharmacological beneficial effects in ethanol abstinence syndrome.

Extraction is a method used for separation of therapeutically desired active constituents with selective solvents and is the first crucial step of the process. It is reported that Soxhlet extraction method is highly efficient in extracting phenolic compounds [249]. The measure of the content of the drug extracted by the particular solvent is called as the extractive value. Plant material was extracted individually with Soxhlet method and the extraction yield was found to be *Bacopa monnieri* (13.75)%, *Hypericum perforatum* (13.25)%, *Centella asiatica* (14.5%), *Withania somnifera* (11.25%), *Camellia sinensis* (13.5%), *Ocimum sanctum* (16%). The individual plant extract obtained then was screened for the qualitative phytochemical evaluation using different phytochemical methods and alkaloids, glycosides, saponin glycosides, coumarin glycosides, anthraquinone glycosides, flavonoids, tannin and phenolic, steroids & triterpenoids, amino acids, carbohydrates and pentoses etc. were found to be present. For the preparation of POL-6 most effective doses of the plants were selected from the literature and POL-6 was prepared by mixing all the plant extracts (*Bacopa monnieri, Hypericum perforatum, Centella asiatica, Withania somnifera, Camellia sinesis and Ocimum sanctum*) in a ratio 2:1:2:2:1:2 proportions respectively. Initially 100g of the POL-6 was prepared by mixing the extracts of *Bacopa monnieri* (20g), *Hypericum perforatum* (10g), *Centella Asiatica* (20g), *Withania somnifera* (20g), *Camellia sinesis* (10g) and *Ocimum*

sanctum (20g). To ensure quality for herbal medicines, the WHO recommends that the plant material should fulfill the Indian Pharmacopoeia specifications [250]. Standardization is an essential parameter in any preparation since it determines the product quality and to avoid any variations. Hence, as a part of standardization process POL-6 was assessed through various parameters which are mentioned in the Indian Pharmacopoeia. Loss on drying is the major factor that causes the deterioration of herbal preparations. This is especially important for materials that degrade quickly in the presence of water. This results in the degradation processes caused by enzymes causing the development of microorganisms [251]. As per Indian Pharmacopoeia limits loss on drying after the exposure to 105^{o}C should be <5%. In our study loss on drying of POL-6 was found to be 4.12 ± 0.21%. Total ash content is the incombustible plant material and is known as the total ash content or ash value. It may compose of metal nitrates, carbonates, phosphates, sulphates, silicates taken up from the environment or soil. It has two types of ash of a) physiological b) non-physiological ash [251]. In accordance to Indian Pharmacopoeia limits the total Ash value should be <10% and the value of acid insoluble ash should be <5%. In our study total Ash value of POL-6 was 9.30 ± 0.93% and Acid Insoluble Ash value was 0.62 ± 0.09%. Bulk density depends upon the spatial arrangement of particles and particle's density in powder and defined as the ratio of mass to the volume of untapped powder including interparticulate void volume. Carr's compressibility index, Hausner's ratio and Angle of repose, are related to interparticle interactions and can be used to determine the flowable properties of the material [252]. As per Indian Pharmacopoeia the Carr's index value is considered to be excellent if it is less than 10%, 11-15% is considered to be good, 16-20% is considered to be fair, 21-25 % is considered to be possible, 26-31% is considered to be very poor and >32% is considered to be very very poor. In our study the Carr's index value of POL-6 was found to be 23.24%. The Indian Pharmacopoeia limits for the angle of repose are, $<25^{o}$ is considered to be excellent, 25-30 is considered to be good, $30\text{-}40^{o}$ is considered to be passable and $>40^{o}$ is considered to be very poor. In our study the angle of repose of POL-6 was found to be 27.2^{o} ± 1.3. From the standardization parameters we concluded that the contents present in the POL-6 are within permissible limits as per Indian Pharmacopoeia and the standardization gives a perspective for further research investigation on POL-6.

Further, POL-6 was exposed to qualitative phytochemical analysis and the presence of secondary metabolites like alkaloids, flavonoids, proteins, glycosides, fixed oils, phenolic, sugars and tannins were evaluated. Detection of the presence of phytochemicals is important

to ascertain their medicinal value [252]. In the previous studies it is reported that flavonoids, play a beneficial role in the ethanol withdrawal syndrome [253,254]. Our study showed that pharmacologically active compounds like carbohydrate, flavonoids, glycosides, alkaloids, steroids, tannin and phenolic are present in the POL-6. Flavonoids are a group of naturally occurring compounds. The biological activity of flavonoids in stimulation and triggering the generation of antioxidant enzymes in human body are well reported [255]. Sever oxidative stress and weakened antioxidant activity was seen in the alcohol withdrawal patients [256]. Quercetin, a flavonoid showed protection against ethanol-induced oxidative stress by enhancing endogenous antioxidant GSH [257]. The flavonoid content quantified in the POL 6 was 481.14±0.34 mg QR/g. Plant based phenolic and flavonoid compounds have an antioxidant activity by scavenging free radicals which thus have a protective effect on ailments associated with oxidative stress. The phenolic content estimated in POL-6 was 316.66±0.28 mg GAE/g. Tannins are reported to reduce the ROS level [258]. The tannin content estimated in POL-6 was 100.43±0.41 mg of CE/g DW. The results from the qualitative estimation of POL-6 showed that POL-6 is rich in flavonoids, tannins and phenolic.

HPLC is an important analytical chromatographic technique used to split a mixture of compounds for qualitative and quantitative analysis [259]. The results from the chemical profiling analysis done by using HPLC showed eleven peaks at the retention time between 1.892 to 30.942 minutes, indicating the presence of eleven major compounds in the POL-6. Polyherbal preparations containing plant extracts of the different plants are reported to have a diverse group of chemical constituents [260-263]. After qualitative phytochemical profiling of POL-6 by HPLC, POL-6 was further tested for mass spectroscopy profiling through Liquid Chromatography-mass spectrometry. LC-MS studies on POL-6 revealed the presence of fourteen major compounds Withaferin A, Withanolide, Luteolin, β-Sitosterol, Quercetin, Bacoside A, Hypericin, Rutin, Linalool, Caffeic acid, Catechin, Eugenol, D-Mannitol and Withanone. Among these fourteen major six compounds were further quantified by using HPTLC. HPTLC was performed for the quantification of Withaferin A, Quercetin, Caffeic acid, β-Sitosterol, Rutin and Catechin in the POL-6. HPTLC study revealed that the Withaferin A was found in the POL-6 at Rf value 0.59 and was 0.921% w/w of POL-6. Withaferin A, an active compound of *Withania somnifera* family (Solanaceae steroidal lactone). It is reported to attenuate the symptoms of ethanol withdrawal induced anxiety, dysphoria and hyper excitability [264]. Quercetin was found in the POL-6 at Rf value 0.65

and was 1.50% w/w of POL-6. Anxiolytic action of Quercetin is reported in the alcohol withdrawal induced by chronic ethanol exposure and quercetin also prevents "reactive oxygen species" production [265,266]. Quercetin also inhibits the seizures which are secondary to the ethanol withdrawal [267]. Caffeic acid was found in the POL-6 at Rf value 0.49 and was 1.059% w/w of POL-6. Caffeic acid is reported to produces anti-depressive and anxiolytic-like effects [268]. Rutin was found in the POL-6 at Rf value 0.86 and was 0.86% w/w of POL-6. Rutin is reported to have protective effect against ethanol-induced neurotoxicity and also have a potent antioxidant activity [269]. β-Sitosterol was found in the POL-6 at Rf value of 0.61 and was 0.60% w/w of POL-6. β sitosterol is a plant sterols having a similar structure to the cholesterol. Previous studies reported the good antioxidant potential of β sitosterol [270]. Catechin was found in the POL-6 at Rf value 0.22 and was 2.86% w/w of POL-6. Catechin is reported as an antioxidant and provides protection against reactive oxygen species [271].

Oxidative stress occurs when there is an imbalance between the Reactive oxygen species generation and antioxidant ability of enzymes in the body is disturbed. There is a production of free radicals implicated in causation of many diseases [272]. Antioxidants are beneficial in relieving oxidative stress by preventing free radical mediated damaging effect on biomolecules such as proteins, DNA and lipids. The alcohol-withdrawal individuals are vulnerable to enhanced formation of reactive oxygen species and ethanol-derived free radicals consequently increase in the oxidative stress [273,274]. Previous studies supports that there is significant reduction of cellular antioxidant defense SOD activity thus increased neurons vulnerability to oxyradical injury. The presence of secondary metabolites in the plant extracts are reported as potent free radical scavenger [275]. Furthermore the presences of diverse group of phytomolecules confirmed in POL-6 have capability to protect against oxidative stress. In the present study antioxidant activity was done by performing DPPH and Nitric oxide assays. DPPH is a free radical and has a spare electron over the molecule which on absorption in ethanol gives deep violet coloration. Change in color occurs when DPPH solution is mixed with a free radical scavenging antioxidant, the violet color changes to yellow [276]. The results of the DPPH antioxidant activity of the POL-6 showed high free radical scavenging properties. The IC_{50} was calculated and for ascorbic acid it is 18±0.18 µg/ml and for POL–6 22.54±0.21 µg/ml. Less IC_{50} value indicated more antioxidant activity. Endothelial cells and macrophages through nitric-oxide synthase produce nitric oxide which show interaction with free radicals and produces the harmful peroxynitrite. The formed peroxynitrite directly causes the oxidation of LDLs, and results in damage to the cell

membrane irreversibly [277]. Nitric oxide form oxides of nitrogen after reacting with oxygen, known as nitrites which are inhibited by the POL-6 showing its antioxidant activity in nitric oxide assay. In nitric oxide assay the IC_{50} value of POL–6 was found to be 21.38±0.17 µg/ml and was comparable to the IC_{50} value of the standard ascorbic acid (17.99 ± 0.17 µg/ml). Present findings showed that POL-6 have a good antioxidant potential. The *in-vitro* findings of the POL-6 give a perspective for its further *in-vivo* investigation.

Safety studies of the polyherbal preparations are required for the evaluation of toxicity and these provide dose selection criteria [278]. Herbal medicines are used by 80% of population but despite common use, there is limited evidence about their safety and efficacy [279]. Much more needs to be done, to develop the standard dosage benchmark and sufficient scientific evidence for herbals and botanicals. Accumulated evidences from safety studies showed few extremely toxic plants *Momordica charantia* [280], *Erythrophleum guinees* [281], *Crocus sativus* [282] and *Urtica dioica* [283]. The safety of herbal plants is must and carried out by doing acute and sub-acute safety studies in rodents and non-human primates [284]. Toxicity means being poisonous, leading to adverse effects caused by the interaction between toxicants and cells. Natural product mixtures are reported to have synergistic interactions and literature also reports that, antagonism, in which effects of active constituents are masked by other compounds in a complex mixture, also occurs in natural product mixtures [285]. POL-6 is a mixture of several compounds so it might be possible that effect of some compounds could have been masked by the other compounds or some compounds might have shown synergistic interactions. Hence it is necessary to assess its safety prior to its utilization. Although, the plants used for the preparation of POL-6 had been traditionally used for curing many ailments and their toxicity studies are well reported but, keeping in view that POL-6 is a mixture of several compounds the *in-vivo* evaluation of toxicological profile of the POL-6 was assessed in the present study. In acute toxicity evaluation POL-6 was given at 2000 mg/kg dosages in Wistar rats. No sign of toxicity or death at this dose (2000 mg/kg, oral) was seen, suggesting a lethal dose 50% (LD_{50}) on oral exposure would be above 2.0 g/kg. These results of acute toxicity showed that in single dose administration, there are no adverse effects indicating that POL-6 will be safe under the dosage of 2000mg/kg. In sub-acute toxicity studies POL-6 was administered orally at doses 250, 500 and 1000 mg/kg for 28 days and the biochemical, hematological and histopathological parameters were monitored. Interestingly no sign of adverse effect were seen. Animal's general behavior, feed consumption, water intake and body weight was observed and in comparison to control no significant changes were found. Changes in water intake, feed consumption, body weight and

general behavior are the marker of injurious effects of drugs [286]. Scientific studies confirmed that increase or decrease pattern in body weights are followed with fat augmentation and physiological variation responses to the plant extracts, not related to any adverse effects of chemicals [287]. Changes in organ weights and organ to body weight ratio may be because of organ damage, that can be a perceptive indicator of toxicity [288]. In present finding, there was no significant difference among treatment and control groups at all tested dose levels. The hematopoietic system is important index of pathological and physiological status and moreover sensitive target site of toxic compounds [289]. It provides information regarding general organs which are at risk to toxic agents. In evaluating toxicity of drugs and plant extracts assessment of liver (SGOT, SGPT, ALP and bilirubin) and kidney function (blood urea and creatinine) is a very vital index and any alterations results in abnormal functioning [290,291]. In the present study, hematological and biochemical parameters between control and drug treated groups, showed that the POL-6 was nontoxic as all parameters were according to the reference ranges. In all groups no significant differences were observed. The findings indicated that the prepared POL-6 formulation was non–toxic as no alteration was seen in biochemical and hematological parameters including the tests that were performed for kidney, liver and lipid metabolism. In toxicity studies the usefulness of organ weight includes their sensitivity that predicts toxicity, physiologic perturbations, and acute injury which correlates with the histological findings [292]. This is further confirmed by the organ histological examination. Interestingly, no necrosis or pathological features was observed in control and POL-6 treatment groups as revealed by internal organs histology suggesting no detrimental changes. The results from the safety studies showed that POL-6 administration did not cause any toxicity or mortality and can be considered as non-toxic.

Chronic alcoholism impose worldwide major public health problem produces both adaptation and neurotoxicity in the brain, resulting in tolerance and dependence. Alcohol dependence results due to excessive ethanol intake, its abruption leads to the ethanol withdrawal syndrome, which includes anxiety, insomnia, delirium, sweating, tremors, convulsions and hypertension [293]. Anxiety, depression and seizures are the most important negative motivators to experience the same level of the rewarding effects of alcohol [294]. Alcohol withdrawal syndrome results in adaptive adjustments in the brain areas such as the amygdala, prefrontal cortex and hippocampus associated with changes in many neuropeptide, neurotransmitter and hormonal systems. Ethanol withdrawal leads to an imbalance in excitatory (especially glutamate,), and hypo function of inhibitory neurotransmitter (especially GABA) receptors involved in the modulation of anxiety and depression. The most

appropriate model for exploring ethanol withdrawal syndrome in animals is the ethanol administration in a liquid diet or a two-bottle choice drinking paradigm model. The latter model is proven to be clinically relevant to mimic the human condition as the animals can voluntarily consume either ethanol or water [295-297]. Previous studies reported dependence and abstinence to alcohol occurs in rats at daily consumption of ethanol over 9 g/kg for 15 days continuously [298]. The two-bottle choice drinking paradigm model was selected in our study. An increase in the body weight of the rats approximately 11.2 % in control group animals and 6.4% in ethanol fed animals was observed over the initial body weight at the end of the study. The body weight in ethanol fed rats changes lightly during the study as compared to the normal control rats. Earlier studies reported that alcohol decreases the secretion of digestive enzymes and affects absorption, metabolism and excretion of essential nutrients [299-301]. Hence alcohol consumption by the alcohol fed animals could be the possible reason of slight change in body weight in comparison to control animals. Ethanol intake by individual rat in ethanol fed groups was also recorded daily during the study and calculated as g/kg/day. Daily ethanol consumption in ethanol fed groups varies between 13.57±1.85 to 17.12±1.34 g/kg during the exposure to 9% ethanol. To see whether the alcohol liquid diet, will achieve reliable BACs and also to confirm clearance of alcohol during withdrawal periods BACs assay was performed. BACs results in our study indicated the complete clearance of ethanol concentration after 72 hour of alcohol withdrawal. Previous studies reported that BACs in the range of 80 to 132 mg % during alcohol intake results in ethanol dependence [302, 303]. In our study BAC was found to be 111.6 ± 8.151 mg % on 15^{th} day which showed ethanol dependence in animals.

We further investigated the effect of POL -6 on anxiety in ethanol withdrawal rats. In present study high level of anxiety was observed on 3^{rd} day of ethanol withdrawal. Hence, we explored the effect of POL-6, diazepam and vehicle, in the EPM and LDT on 18^{th} day only. EPM and LDT are well known tests used for exploring the antianxiety effects of the drugs [304]. EPM is the most widely used apparatus used to assess exploration, anxiolytic responses and motor behavior. Rodents display approach avoidance conflict which is stronger in exposed open areas and preference to enclosed arm, therefore, spending more time in enclosed arm. When animal enters the open arm they freeze and show fear [305]. Drugs that relieve anxiety increase the time spent and number of entries in open arms. In the present study, disease (ethanol withdrawal) control group animals spent less time in the open arms exploration, and the time spent in closed arms was more as compared to normal control

animals. POL-6 (20, 50 and 100 mg/kg, oral) and diazepam (2mg/kg) treatment given for following 3 days increased the number of entries and the time spent by the alcohol fed rats in the open arm. This show the anxiolytic effects of the POL-6 in the rats. LDT test is useful model to study anxiolytic activity. The brightly lit compartment represents a destructive environmental stressor that reduces the normal exploratory behavior of rats. Exploratory behavior from one compartment to other and the time spent in each compartment are parameter reported in anxiety. In the present study there was an inhibition of anxiety behavior, latency to the dark chamber by the animals was decreased, and numbers of transitions in both the compartments and time spent in light chamber were also decreased showing the anxiety amid alcohol withdrawal. Treatment with POL-6 (20, 50 and 100 mg/kg, oral) and diazepam (2mg/kg) for following 3 days increases the time spent and number of entries in the light compartment by the rats. The neurochemical changes in GABAergic system of brain is responsible for anxiety like behavior, resulting in increased behavioral excitability [306]. During ethanol withdrawal, there is decline in the inhibition of excitatory activity by $GABA_A$ receptors that leads to CNS hyper excitability [307]. Therefore, stimulation at normal level even can cause over-excitation due to the reduced suppression of the CNS, thus the alcohol withdrawal induced anxiety was observed. The individual constituents present in the POL-6 have been reported previously for their antianxiety activity at high doses, then the need for the development polyherbal preparation lies in Ayurveda which states that when the drugs are combined, shows the potentiation of response and ameliorated the associated side effect of individual drugs [308]. POL-6 might have potentiated the anxiolytic action as *Withania somnifera* is proven to have GABA mimetic activity [309], Moreover, *Withania somnifera* has evidences to reduce the levels of mediators that causes anxiety [310,311]. Adenosine has also been reported in the development of ethanol withdrawal syndrome. *Camellia sinensis* has stimulating effect on brain due to its methyl xanthine content that antagonizes adenosine thus ameliorating ethanol withdrawal state [312,313]. *Ocimum sanctum* also reported to decrease the levels of anxiogenic mediators in brain also called as antistressor and reported to possess antioxidant activity [314-316]. Thus we can conclude that herb-herb combination in the POL-6 proven to be beneficial in ameliorating alcohol withdrawal anxiety in rats.

Depression is prevalent neuropsychiatric disorder that affects individual's quality of life and characterized by change in mood, sleep disturbances, lack of interest in surroundings, loss of libido, loss of appetite [317]. According to WHO depression is one of the burdensome

diseases of society [318]. Epidemiological literature supports a strong positive association drinking alcohol and depression [319-321]. Most of the anti-depressants are not effective in alcohol withdrawal related depression, thus novel ethanol withdrawal antidepressants, could be a key in improving the cessation rate [322,323]. The clinical and experimental is based upon the finding that ethanol-induced depression was associated with increased expression corticotropin releasing factor (CRF) and reduced expression of NPY neuropeptide Y (NPY) systems in the brain [324]. Another study also reported that hippocampal brain-derived neurotropic factor (BDNF) which is responsible for neuronal development, plasticity and survival was reduced following withdrawal from daily intake. Additionally, in abstinence phase there is reduced CSF 5-hydroxyindoleacetic acid that reduces 5-HT synthesis and tryptophan uptake contributing to the depressive states [325]. The FST and TST are widely used tests for antidepressants screening [326]. In the FST rat is placed in a cylinder of water from which it cannot escape followed by the initial struggling period, climbing and swimming and lastly displays a floating or immobile posture [327]. In the TST test, rat is suspended by its tail and immobility is scored. In the present study, normal control group rats displayed increased immobility in the forced swim test and tail suspension test during 3rd day of withdrawal from ethanol. These results are supported by previous findings describing that ethanol withdrawal induces depressive like effects in rats [328,329]. POL-6 (20, 50 and 100 mg/kg, oral) and fluoxetine (5mg/kg) treatment for following 3 days in ethanol withdrawal rats showed a declination in immobility time in FST and TST. This shows the antidepressant effect of POL-6 in alcohol withdrawal animals. Previously reported preclinical studies indicate that *Bacopa monnieri [330]*, *Withania somnifera* [331,332], *Camellia sinensis* [333], *Hypericum perforatum* [334] extracts normalized the reduction of BDNF mRNA levels in the rat brain hippocampus that may underline their ability as an antidepressant agent. Thus we can conclude that herb-herb combination in the POL-6 has been proven to be beneficial in ameliorating the depressive like behavior in alcohol withdrawal rats.

Ethanol withdrawal following chronic intake results in relapsing disturbances involves symptoms such as neural excitation (seizures), depression and anxiety [335]. In rodents acoustic stimuli easily triggers epileptic seizures during ethanol withdrawal [336]. Alcohol withdrawal seizures (AWS) are a serious, life-threatening problem. There is progressive increase in tonic–clonic seizures with single successive detoxification [337-339]. In the previous studies 25% cases reported that AWS are the trigger for status epilepticus and temporal lobe epilepsy [340-342]. The neural networks in the brain stem and neocortex

region including inferior colliculus are associated with AWS. Thus, when the person withdraws alcohol and its potentiating effects are diminished, the reduction in synaptic $GABA_A$ inhibitory function triggers the withdrawal seizures in humans [343]. Another mechanism that contributes is T-type calcium channels activation and function was found to be enhanced leading to hyper excitability observed during alcohol withdrawal [344]. Kokka et al., 2015 [345] showed that rats undergoing withdrawal showed increased sensitivity and persistent reduction in seizure threshold to the convulsant drug PTZ. Previous literature reported that the administration of sub convulsive dose to normal rat did not produce any convulsive behavior [346]. A pentylenetetrazole (30 mg/kg) subconvulsive dosage was given in the alcohol withdrawal followed by the observation period of 30 min for the convulsive behavior. Animals entering into convulsive phase show loss of postural control, myoclonic ear facial and body jerks [347]. In normal rats PTZ (30 mg/kg) subconvulsive dosage results in no convulsions (0% convulsion) whereas, ethanol withdrawal rats showed convulsive behavior. Treatment with POL-6 (20, 50 and 100 mg/kg, oral) and Diazepam (2mg/kg) for following 3 days decreased seizure severity and frequency in the alcohol fed animals. This shows the beneficial effects of POL-6 in ethanol withdrawal seizures.

Locomotor activity is governed by the dopamine receptors [348]. During the phase of ethanol withdrawal there is profound decrease in the activity of mesolimbic DA neurons and this reduced dopamine function contributes to withdrawal symptoms including hyper excitability [349,350]. In alcohol withdrawal; NMDA receptors are involved in neuroplasticity, a process characterized by neural reorganization that likely contributes to hyper excitability [351]. In our study ethanol abstinence was characterized by increased hyper excitability of rats in the actophotometer which was attenuated by POL-6.

Hematological parameters evaluation indicates the extent of damaging effects of foreign compounds such as plant extract on blood constituents of the animals. It also provides information about the status of bone marrow activity and hemolysis. In regards to hematological analysis most of the values were normal in comparison with the control group. Furthermore blood chemical analysis was also done to evaluate any toxic consequences on blood glucose, lipid profile and on liver and kidney function tests. GGT, SGOT, SGPT, are utilized in the clinical practice as traditional alcohol markers to identify chronic heavy drinking [352]. Excessive alcohol consumption is reported to increase the GGT level in the serum that is one of the cause of oxidative stress [353]. In our antioxidant studies POL-6 showed good antioxidant activity and all the plants used for the preparation of POL-6 have

reported antioxidant properties. In our studies we have quantified the six major compounds through HPTLC in POL-6. These all compounds Quercetin, Withferin A, β-setosterol, Catechin, Rutin and Caffeic acid are reported to reduce oxidative stress. Hence, the potential antioxidant property of POL-6 might have brought about the inversion of all the mentioned alcohol biomarkers.

Further we explored the influences of POL-6 on the mRNA expression of $GABA_A$ receptor subunits. $GABA_A$ receptor allosteric binding sites are targets for alcohol that modulate GABAergic function [354,356]. Alcohol targets $GABA_A$ receptors because they are key inhibitory neurotransmitters in the CNS and play a central role in mediating the consequences of ethanol [357,358]. Decreased GABAergic neurotransmission is reported in the alcoholics and alcohol dependence individuals [359,360]. $GABA_A$ receptors have a number of subunit isoforms generally α1- α5 subunits. The gene which encodes the Gabra (1-5) subunits is reported to have a remarkable plasticity in alcohol addiction [361-364]. Reduction in GABAergic transmission and $GABA_A$ receptors down regulation have been reported in alcohol withdrawal induced symptoms [365,366]. Alcohol withdrawal is reported to decrease the mRNA expression α1- α5 subunits of $GABA_A$ receptor in amygdala, prefrontal cortex and hippocampus which is the major cause of negative withdrawal effects [367]. $GABA_A$ receptors down regulation in alcohol withdrawal develops a hyperglutamatergic state, which in combination with reduced GABA function leads to the excessive excitatory signaling, resulting to the alcohol withdrawal syndrome [368,369]. In alcohol withdrawal GABA levels have also been reported to be low in the cerebrospinal fluid of humans [370]. In our study, we found that alcohol withdrawal amid 15 days of alcohol utilization in rats emanated a decrease of Gabra1, Gabra2, Gabra3, Gabra4 and Gabra5 gene expression in prefrontal cortex, hippocampus and amygdala of the rats. Interestingly, treatment with POL-6 (20, 50 and 100 mg/kg, oral) for following 3 days normalized the down regulated Gabra1, Gabra2, Gabra3 and Gabra5 gene expression in the prefrontal cortex; Gabra1, Gabra 2, Gabra 3 and Gabra 4 gene expression in the amygdala; Gabra2, Gabra3 and Gabra5 gene expression in the hippocampus of the rats. However no effect was seen on the down regulated gene expression of Gabra4 in the prefrontal cortex, Gabra5 in the amygdala and Gabra1 and Gabra4 in the hippocampus of the rats with any of the POL-6 treatment. In an another group treatment with Diazepam (2mg/kg) for following 3 days normalized the down regulated Gabra1, Gabra2, Gabra4 and Gabra5 gene expression in prefrontal cortex, Gabra1, Gabra2, Gabra3 and Gabra5 gene expression in amygdala, Gabra1, Gabra 2, Gabra3, Gabra4 and Gabra5 gene

expression in the hippocampus of the rats. However no effect was seen on the down regulated gene expression of Gabra3 in prefrontal cortex, Gabra4 in amygdala of the rats with the diazepam treatment. *Withania somnifera*, *Hypericum perforatum*, *Oscimum sanctum*, *Camellia sinesis* are reported to have GABA mimetic effects [371-373]. The constituents present in POL-6 are also reported to modulate GABAergic functions. Quercetin is reported to regulate GABAergic transmission in the prefrontal cortex [374]. Rutin is reported to modulate $GABA_A$ receptors and increase GABAergic neurotransmission in the amygdala [375]. Withferin A is reported to have GABAergic activity [376]. Catechin is reported to modulate GABAergic neurotransmission [377]. GABA mimetic effects of the constituents present in POL-6 may have caused normalization of genes of $GABA_A$ receptor subunits in ethanol withdrawal. Our findings may conclude that GABA mimetic effects of POL-6 may be beneficial in normalizing Gabra1, Gabra2, Gabra3, Gabra4 and Gabra5 genes of $GABA_A$ receptor subunits in rats' amygdala, prefrontal cortex, and hippocampus.

During alcohol withdrawal, decreased GABA activity and the increased effect of glutamate are related to each other. When interacting with NMDA receptors, glutamatergic neurons are exposed to GABAergic effect through $GABA_A$ receptors [378]. Hence we further explored the effects of POL-6 on the mRNA expression of NMDA receptor subunits. NMDA receptor is a major target of alcohol in the brain. Role of NMDA receptors in ethanol withdrawal, dependence and craving is well established [379]. NMDA receptors are comprise of GluN1, GluN2 (2A–D) and GluN3 subunits [380,381]. It is believed that chronic ethanol exposure and withdrawal leads to the hyper excitability of the NMDA receptor channel due to an increase in the mRNA and protein levels of NMDA receptor subunits. Previous studies reported that ethanol withdrawal up regulated the mRNA level of the NR1, NR2A and NR2B subunits [382-384]. Based on previous literature reports; Grin1, Grin2a and Grin2b genes of the NMDA receptor subunits were explored in the present study [385]. During study, we observed that alcohol withdrawal amid 15 days of consumption emanated an increased relative expression of Grin1, Grin2a and Grin2b in prefrontal cortex, hippocampus and amygdala of the rats. Interestingly, treatment with POL-6 (20, 50 and 100 mg/kg, oral) for following 3 days reversed the up regulated genes Grin1 and Grin2a gene expression in prefrontal cortex, Grin1 and Grin2a gene expression in the amygdala, Grin1 gene expression in the rats' hippocampus. However no effect was seen on the up regulated Grin2a expression in the prefrontal cortex, Grin2b in the amygdala and Grin2a and grin2b expression in the rats' hippocampus with any of the POL-6 treatment. In another group treatment with Diazepam

(2mg/kg) for following 3 days reversed the up regulated Grin1, Grin2a and Grin2 expression in rat's prefrontal cortex, amygdala and hippocampus. *Withania somnifera* is reported to potentiate the NMDA receptors in the hippocampus [386]. *Bacopa monnieri* modulates the NMDA receptor subunits [387]. *Centella asiatica* modulates NMDA receptor subunit expressions [388]. The constituents present in POL-6 are also reported to modulate NMDA receptor functions. Quercetin and rutin are reported to modulate NMDA receptor expressions [389]. Our findings suggest that during ethanol withdrawal; NMDA receptor modulating properties of the constituents present in POL-6 may have normalized the elevated expression of NMDA receptor subunits (Grin1, Grin2a, Grin2b) in rats' hippocampus, prefrontal cortex and amygdala.

SUMMARY AND CONCLUSION

In the present study a polyherbal preparation named POL-6 was prepared by mixing the dried extracts of six plants *Bacopa monnieri, Hypericum perforatum, Centella asiatica, Withania somnifera, Camellia sinesis and Ocimum sanctum* in the proportion 2:1:2:2:1:2 respectively. The ratios of the plants were selected from the literature. The most effective doses of the plants were considered to make the proportion of the polyherbal preparation. Polyherbal preparation was further standardized by studying its organoleptic, physiochemical and physical characteristics as per Indian Pharmacopeia standards. The phytochemical screening of the POL-6 showed the presence of bioactive constituents like flavonoids, alkaloids, glycosides, phenolic and tannins. Phytochemical profiling of POL-6 with HPLC showed the presence of 11 major compounds which was further identified through LC-MS studies. Six major compounds Withferin A, Quercetin, β-sitosterol, Caffeic acid, Catechin and Rutin were further quantified by HPTLC study. POL-6 was further evaluated for *in-vitro* antioxidant activities using DPPH, nitric oxide radical scavenging assays. The alcohol-withdrawal enhances the formation of reactive oxygen species and ethanol-derived free radicals consequently increase in the oxidative stress. POL-6 showed good antioxidant activities and the results from the *in-vitro* findings of the POL-6 gave us a perspective for its further *in-vivo* investigation. Safety studies of the polyherbal preparations are needed to evaluate their toxicity and provide criteria for the selection of a safe dose. Acute toxicity and sub-acute toxicity study of POL-6 was conducted as per OECD 423 guidelines. Safety studies showed that POL-6 is nontoxic in nature and 2000mg/kg in acute toxicity studies and 1000mg/kg in 28 days repeated oral toxicity studies were considered as the no observed adverse effect levels (NOAEL) of POL-6. The effect of POL-6 was further explored on the alcohol deprivation effects following long term voluntary alcohol consumption in rats. Two-bottle choice drinking paradigm model was used for the study giving animals' free choice between alcohol and water for 15 days. Alcohol was withdrawn on 16^{th} day. POL-6, Diazepam and fluoxetine treatment was given on withdrawal days. Influences of POL-6 on the alcohol deprivation effects were further studied on the EPM, LDT and locomotor activity. POL-6 showed protective effects on all the tested behavioral parameters. For studying the effects of POL-6 on ethanol withdrawal seizures a pentylenetetrazole (30 mg/kg) subconvulsive dosage was given in the withdrawal period. POL-6 protects the animals from the seizures showing its beneficial effects in alcohol withdrawal. POL-6 normalized the locomotor hyperactivity during the alcohol withdrawal. Effect of POL-6 was further tested on the GGT, SGOT and

SGPT which are considered to be alcohol markers for identifying chronic heavy drinking. POL-6 showed positives effects on all the alcohol biomarkers showing its potential antioxidant properties which were confirmed earlier on the *in-vitro* antioxidant assays. The potential mechanism through which POL-6 demonstrated antianxiety, antidepressant and other positive effects on alcohol withdrawal symptoms was further explored by studying the influences of POL-6 on the mRNA expression of $GABA_A$ and NMDA receptor subunits. In gene expression studies POL-6 showed GABA mimetic and NMDA receptor modulating properties. The results from the present finding showed that POL-6 possess protective effect in alcohol withdrawal anxiety, depression, seizures and locomotor hyperactivity in rats. Gene expression studies on the isolated brain tissues showed that POL-6 normalizes the GABAergic and NMDA transmission in the amygdala, pre-frontal cortex and hippocampus of the rats and inhibits the ethanol withdrawal behaviors. Therefore we concluded that POL-6 may have therapeutic potential for treating ethanol-type dependence as it suppresses ethanol withdrawal signs and symptoms.

FUTURE PROSPECTS

1. POL-6 may be further compared with its individual constituent herbs for determining the synergistic effect of the constituent herbs.
2. Chronic toxicity studies may be performed on POL-6 for further exploration of its long term beneficial effects.
3. POL-6 may be tested for its more inhibitory effects on the reuptake of neurotransmitters such as noradrenaline, serotonin and dopamine.

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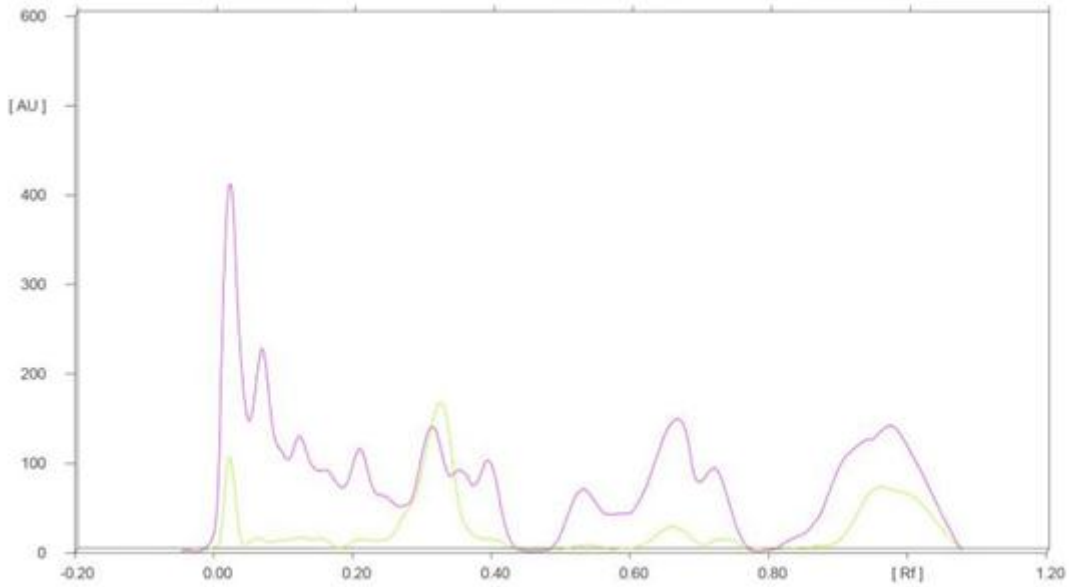
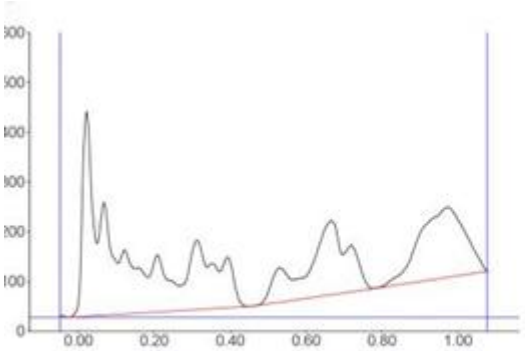
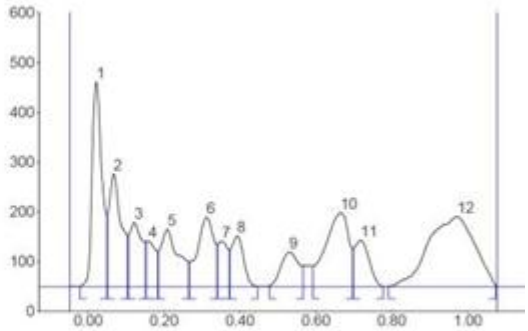
APPENDIX

Withferin A







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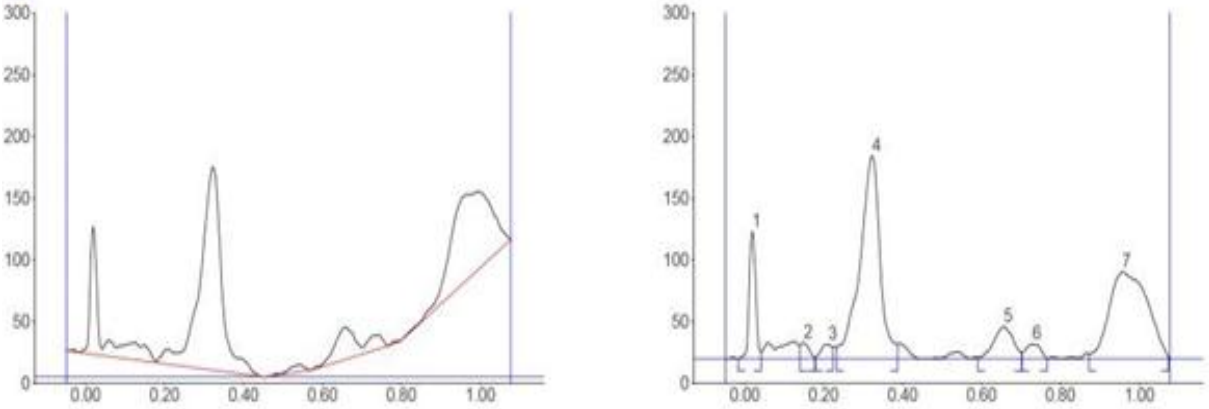
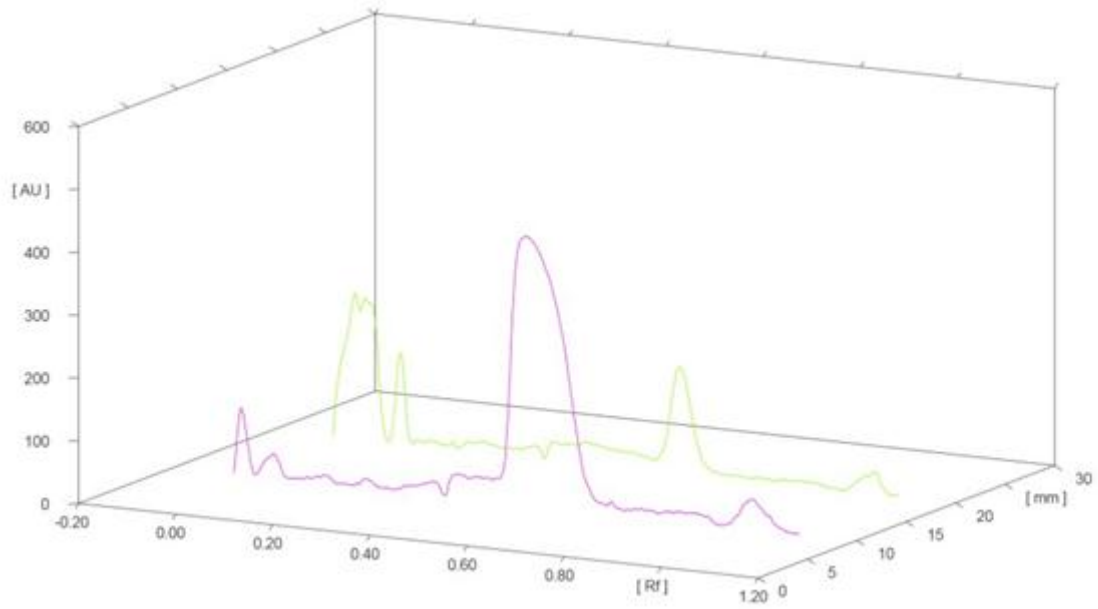
2	0.05	147.8	0.07	226.4	12.90	0.10	103.0	5387.7	9.96	unknown *
3	0.11	103.3	0.12	128.7	7.34	0.15	89.9	3144.0	5.81	unknown *
4	0.15	90.0	0.16	91.0	5.19	0.18	71.3	1658.5	3.07	unknown *
5	0.19	71.7	0.21	114.6	6.53	0.27	50.1	3934.1	7.27	unknown *
6	0.27	50.2	0.31	139.3	7.94	0.34	84.4	4365.2	8.07	unknown *
7	0.34	85.0	0.35	90.9	5.18	0.37	73.9	1699.5	3.14	unknown *
8	0.37	74.2	0.39	101.5	5.78	0.45	0.3	2353.9	4.35	unknown *
9	0.48	1.3	0.53	69.6	3.97	0.57	41.6	2357.6	4.36	unknown *
10	0.59	42.1	0.67	148.1	8.44	0.70	77.0	6535.4	12.08	unknown *
11	0.70	77.2	0.72	93.0	5.30	0.78	0.1	2572.1	4.75	unknown *
12	0.79	0.7	0.97	140.4	8.00	1.08	3.1	12315.9	22.76	unknown *

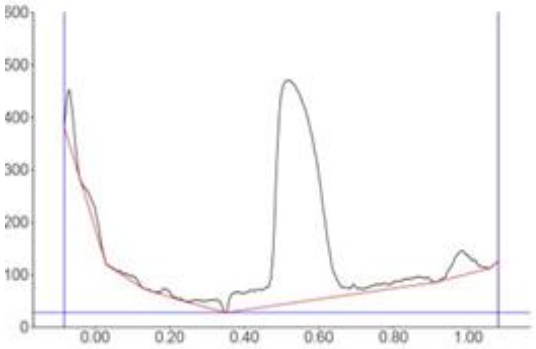
Track 2, ID: Withaferin-A

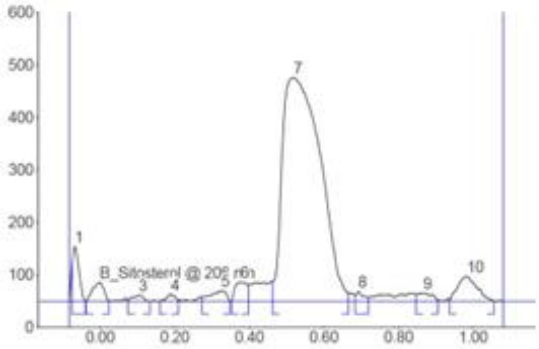


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.02	0.0	0.02	103.2	25.72	0.04	5.6	1221.5	8.76	unknown *
2	0.14	11.1	0.15	13.0	3.24	0.18	1.1	212.2	1.52	unknown *
3	0.18	1.5	0.21	11.9	2.97	0.22	9.6	237.7	1.71	unknown *
4	0.23	9.9	0.32	164.5	41.00	0.39	12.0	6047.7	43.39	unknown *
5	0.59	1.2	0.66	26.1	6.50	0.70	5.3	933.4	6.70	unknown *
6	0.70	5.5	0.73	12.2	3.03	0.77	0.1	333.5	2.39	unknown *
7	0.87	4.4	0.96	70.4	17.54	1.08	2.3	4950.7	35.52	unknown *

β-Sitosterol







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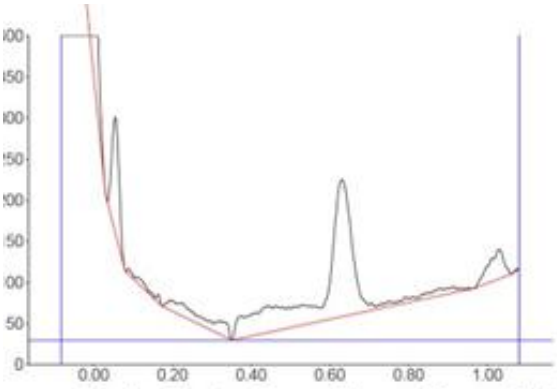
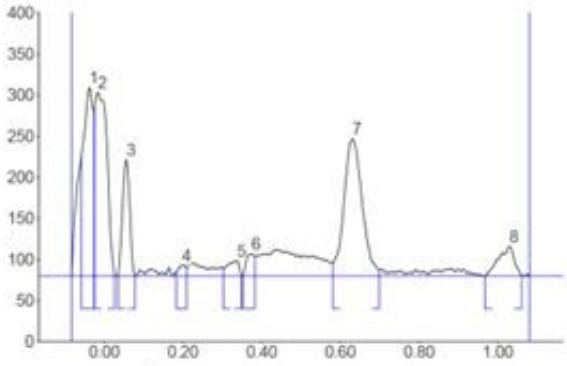
1	-0.08	74.7	-0.07	103.9	14.27	-0.04	0.4	1208.4	3.31	unknown *
2	-0.04	0.7	-0.00	35.9	4.94	0.02	1.4	750.6	2.06	B_Sitosterol
3	0.08	3.6	0.10	11.3	1.55	0.13	0.1	221.7	0.61	unknown *
4	0.16	0.4	0.19	12.6	1.73	0.21	1.5	203.9	0.56	unknown *
5	0.27	7.5	0.32	20.0	2.75	0.35	0.5	593.8	1.63	unknown *
6	0.35	1.4	0.38	36.5	5.01	0.40	31.2	832.0	2.28	unknown *
7	0.46	36.5	0.52	426.1	58.54	0.67	15.6	30284.1	82.96	unknown *
8	0.69	11.7	0.69	19.0	2.60	0.72	6.9	261.7	0.72	unknown *
9	0.85	13.4	0.87	15.6	2.14	0.91	3.2	438.9	1.20	unknown *
10	0.94	4.0	0.99	47.1	6.47	1.06	0.3	1710.0	4.68	unknown *





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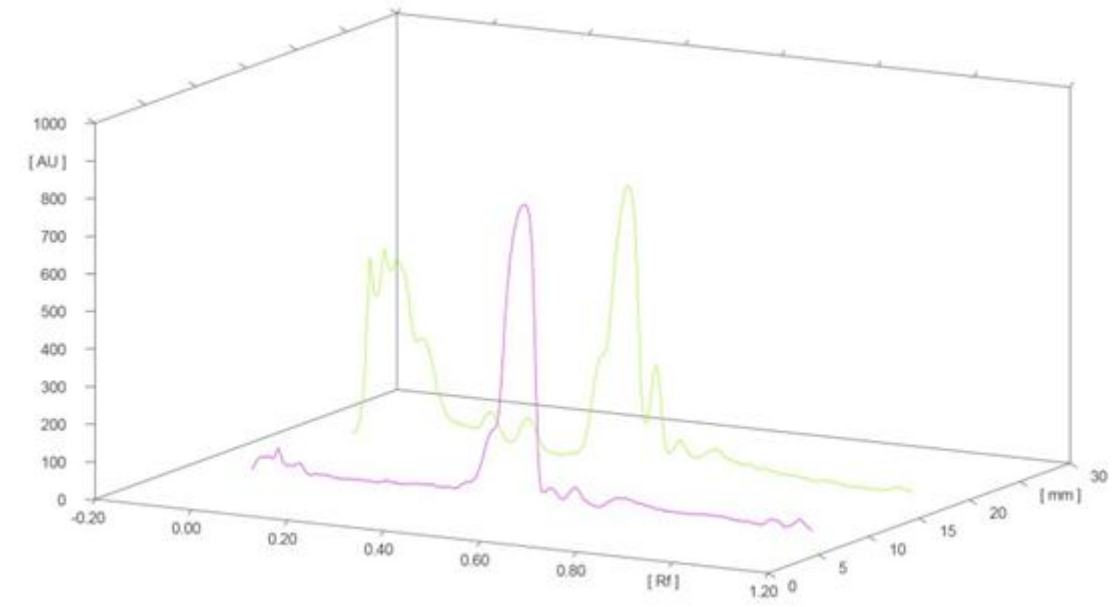
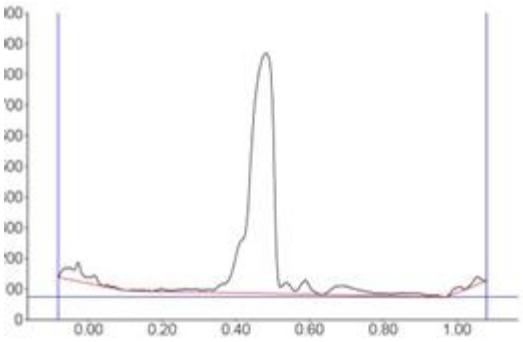
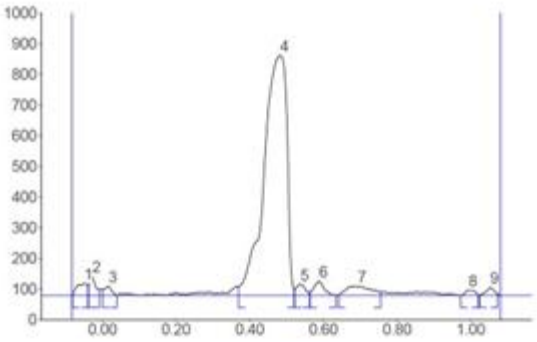
1	-0.06	146.5	-0.04	229.5	26.85	-0.03	202.3	3922.5	21.93	unknown *
2	-0.03	205.0	-0.02	223.2	26.12	0.03	1.7	4809.0	26.89	unknown *
3	0.04	1.7	0.06	141.2	16.52	0.08	0.1	1808.0	10.11	unknown *
4	0.18	5.2	0.20	13.0	1.52	0.21	11.1	181.5	1.01	unknown *
5	0.30	9.2	0.34	18.2	2.13	0.35	0.5	384.9	2.15	unknown *
6	0.35	0.5	0.38	27.0	3.16	0.38	24.5	385.2	2.15	unknown *
7	0.58	16.2	0.63	166.7	19.51	0.70	7.1	5391.1	30.14	unknown *
8	0.97	0.2	1.03	35.8	4.19	1.06	0.5	1002.5	5.61	unknown *

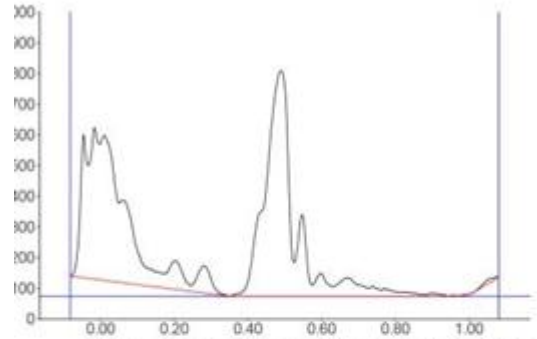
Caffeic Acid

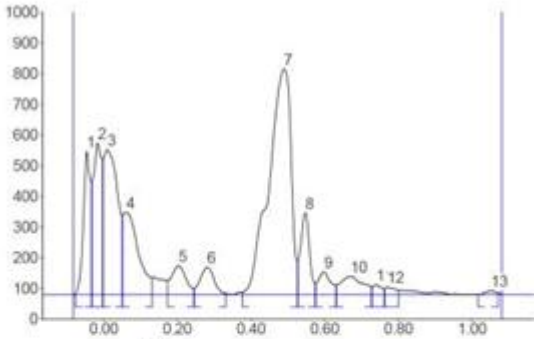






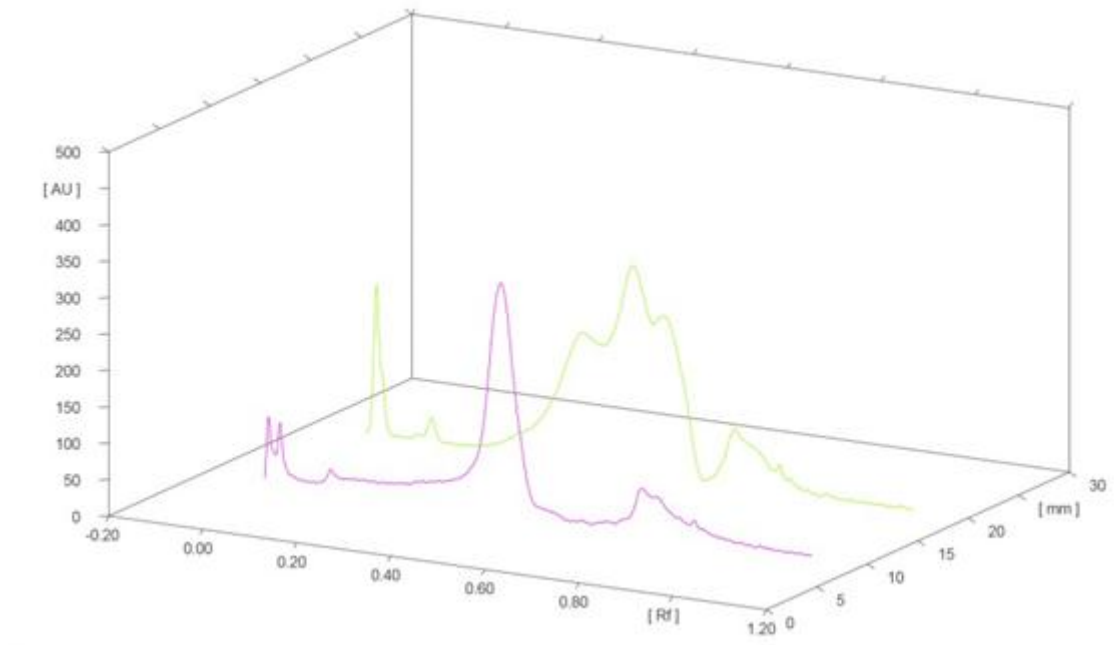
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.08	2.2	-0.05	38.9	3.65	-0.04	34.3	759.6	1.94	unknown *
2	-0.04	34.6	-0.03	61.7	5.80	-0.01	18.6	717.6	1.83	unknown *
3	0.00	20.2	0.02	29.6	2.78	0.04	0.8	413.1	1.05	unknown *
4	0.37	29.3	0.48	782.6	73.49	0.52	21.8	33775.1	86.05	unknown *
5	0.52	22.0	0.54	35.1	3.30	0.56	10.4	619.2	1.58	unknown *
6	0.56	10.5	0.59	45.6	4.29	0.63	0.2	857.3	2.18	unknown *
7	0.64	0.3	0.69	29.5	2.77	0.76	11.1	1423.2	3.63	unknown *
8	0.98	0.3	1.00	16.7	1.57	1.02	0.7	309.1	0.79	unknown *
9	1.03	0.1	1.05	25.1	2.36	1.08	5.3	377.7	0.96	unknown *

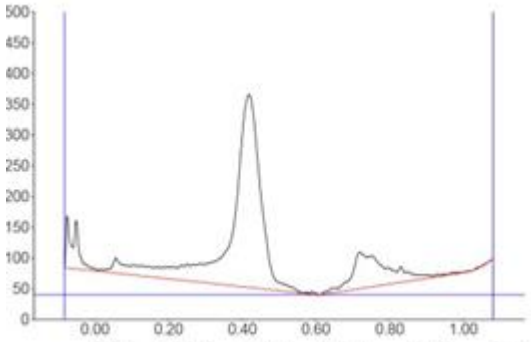


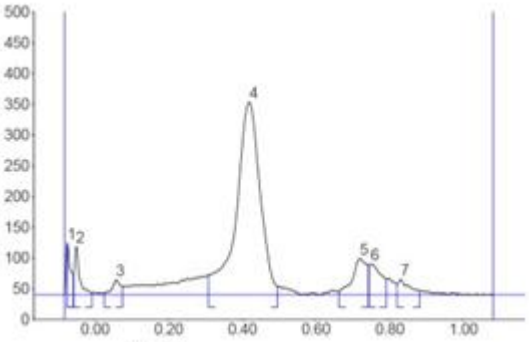


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.08	2.2	-0.05	467.6	15.12	-0.03	369.2	6307.8	7.82	unknown *
2	-0.03	371.6	-0.02	494.2	15.98	-0.00	442.0	7955.3	9.86	unknown *
3	-0.00	442.3	0.01	472.9	15.29	0.05	259.2	12724.2	15.77	unknown *
4	0.05	261.1	0.06	268.8	8.69	0.13	55.3	7888.4	9.78	unknown *
5	0.17	47.1	0.20	94.1	3.04	0.25	19.7	2641.8	3.27	unknown *
6	0.25	20.6	0.28	89.3	2.89	0.33	3.7	2350.4	2.91	unknown *
7	0.38	8.6	0.49	735.0	23.76	0.53	110.1	30779.0	38.15	unknown *
8	0.53	112.2	0.55	266.3	8.61	0.57	37.0	4531.4	5.62	unknown *
9	0.58	38.0	0.60	74.3	2.40	0.63	31.3	1742.2	2.16	unknown *
10	0.63	31.4	0.67	59.2	1.91	0.73	26.4	2497.5	3.10	unknown *
11	0.73	26.6	0.74	32.8	1.06	0.76	18.5	551.2	0.68	unknown *
12	0.76	19.1	0.77	25.6	0.83	0.80	14.2	462.0	0.57	unknown *
13	1.02	0.2	1.05	13.1	0.42	1.07	6.3	253.8	0.31	unknown *

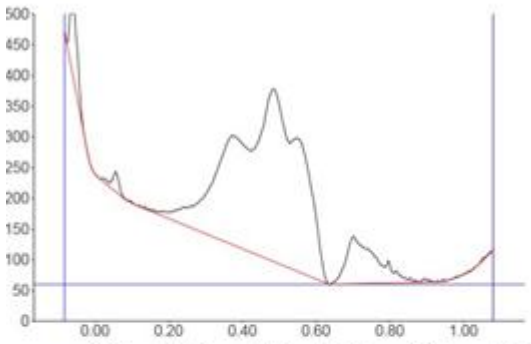
Catechin

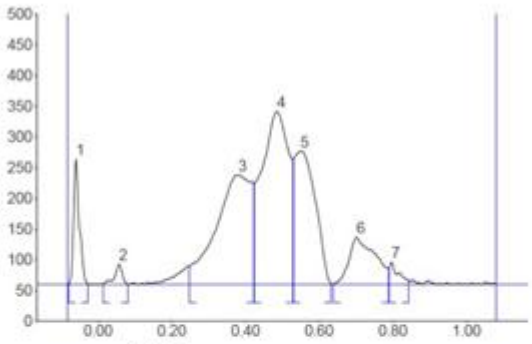






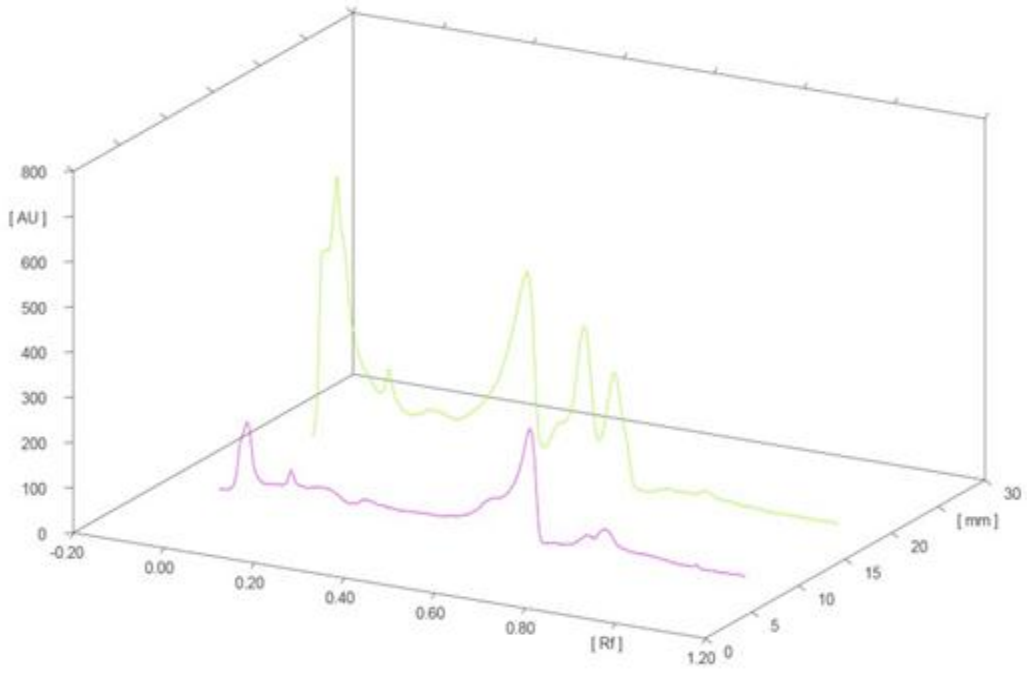
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.08	84.5	-0.08	84.5	13.29	-0.06	33.9	493.1	2.60	unknown *
2	-0.06	35.0	-0.05	78.4	12.33	-0.01	3.4	750.8	3.95	unknown *
3	0.03	3.0	0.06	24.6	3.86	0.08	14.2	392.1	2.06	unknown *
4	0.31	32.4	0.42	313.5	49.32	0.50	13.7	14052.1	73.97	unknown *
5	0.66	5.4	0.72	59.0	9.28	0.74	48.8	1613.1	8.49	unknown *
6	0.75	49.1	0.75	50.1	7.89	0.79	26.2	1146.4	6.03	unknown *
7	0.82	17.9	0.83	25.5	4.01	0.88	6.5	549.7	2.89	unknown *

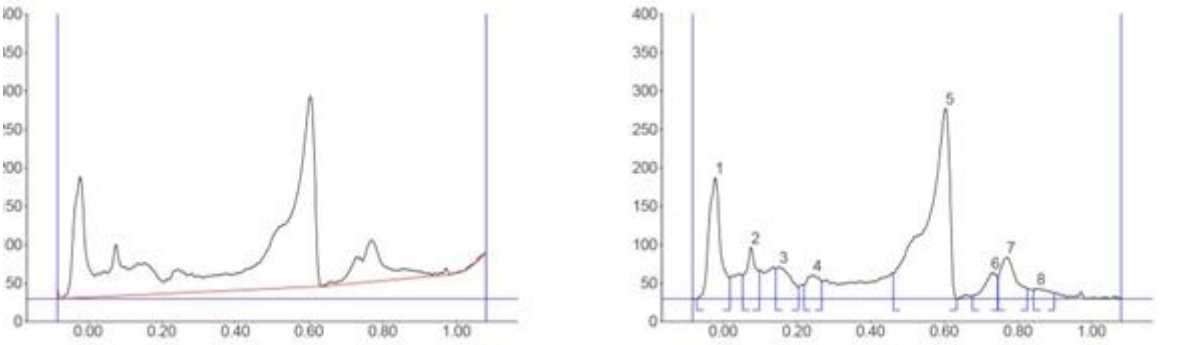




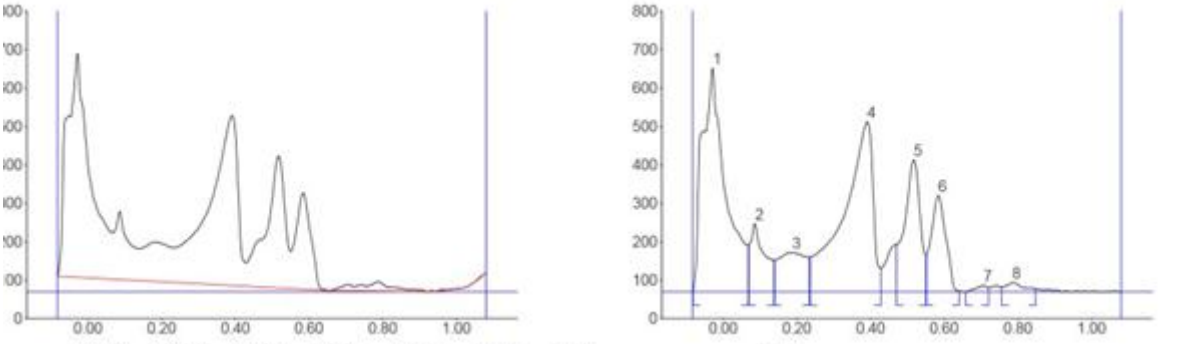
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.08	1.2	-0.06	204.0	19.87	-0.03	0.8	2186.4	5.20	unknown *
2	0.01	0.2	0.06	32.8	3.19	0.08	0.2	463.3	1.10	unknown *
3	0.25	29.1	0.38	178.1	17.35	0.42	165.3	11887.0	28.28	unknown *
4	0.43	165.4	0.49	281.4	27.42	0.53	204.0	14494.8	34.48	unknown *
5	0.53	204.8	0.55	216.7	21.11	0.63	0.7	8626.5	20.52	unknown *
6	0.64	0.7	0.70	77.2	7.52	0.79	26.5	3774.2	8.98	unknown *
7	0.79	26.9	0.80	36.4	3.54	0.85	6.6	600.1	1.43	unknown *

Quercetin



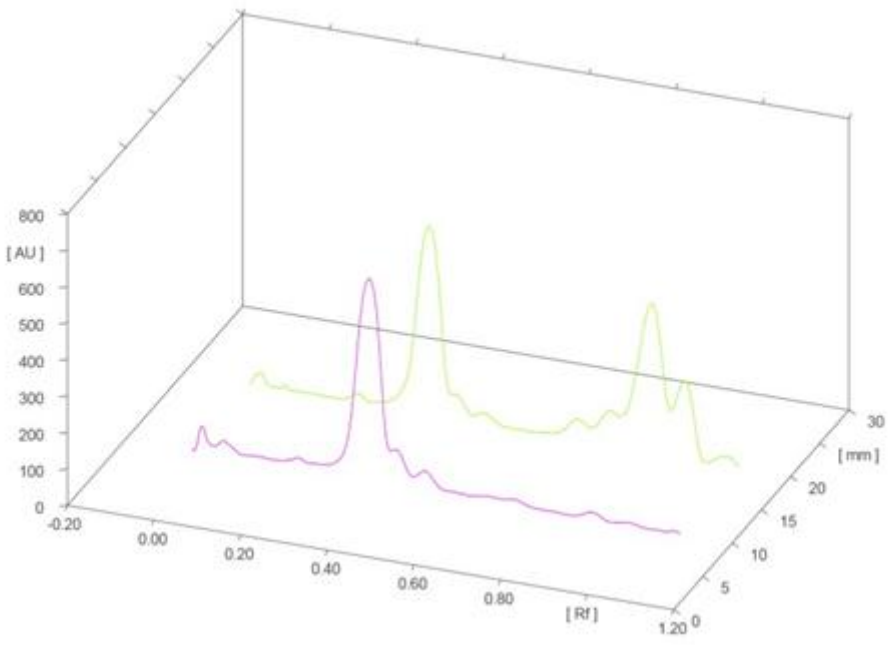


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.07	0.0	-0.02	157.4	24.43	0.02	26.9	3432.0	17.24	unknown *
2	0.05	30.4	0.08	66.5	10.32	0.10	36.6	1238.0	6.22	unknown *
3	0.14	39.3	0.15	41.3	6.41	0.20	15.2	1172.1	5.89	unknown *
4	0.22	18.1	0.25	30.6	4.75	0.27	22.4	790.7	3.97	unknown *
5	0.46	33.7	0.61	247.8	38.44	0.63	0.2	10536.7	52.93	unknown *
6	0.68	4.0	0.73	33.7	5.24	0.75	27.7	775.8	3.90	unknown *
7	0.75	28.2	0.77	53.9	8.36	0.83	13.0	1586.0	7.97	unknown *
8	0.85	11.0	0.85	13.3	2.06	0.90	7.3	376.3	1.89	unknown *

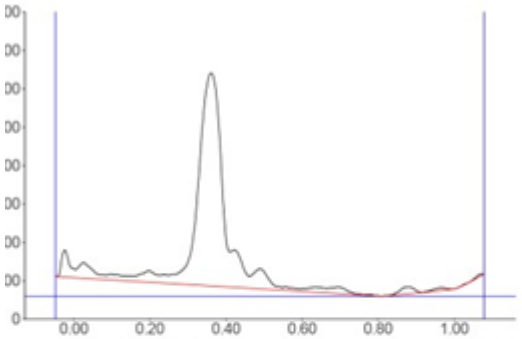


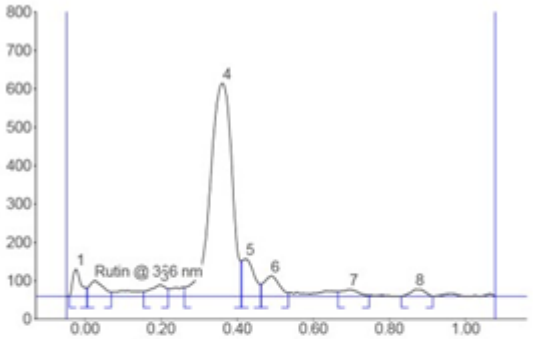
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.08	4.3	-0.03	582.2	30.05	0.07	121.2	25843.0	32.69	unknown *
2	0.07	121.6	0.09	176.7	9.12	0.14	83.0	4864.3	6.15	unknown *
3	0.14	83.1	0.19	102.1	5.27	0.23	89.9	5489.8	6.94	unknown *
4	0.24	90.2	0.39	442.2	22.82	0.43	61.3	24477.2	30.96	unknown *
5	0.47	122.8	0.52	343.2	17.72	0.55	96.4	10233.8	12.95	unknown *
6	0.55	97.2	0.58	250.4	12.92	0.64	1.7	6961.5	8.81	unknown *
7	0.66	0.4	0.71	16.7	0.86	0.72	11.0	352.9	0.45	unknown *
8	0.76	11.3	0.79	23.9	1.23	0.85	7.9	829.1	1.05	unknown *

Rutin

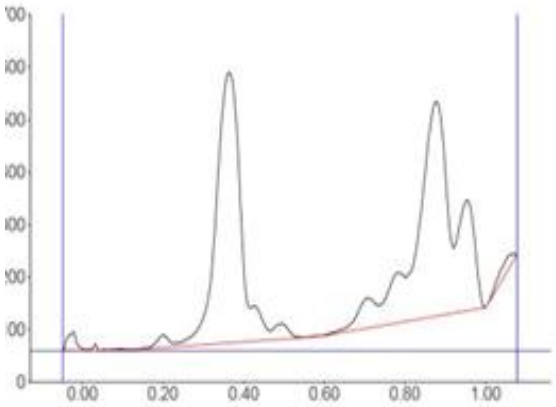


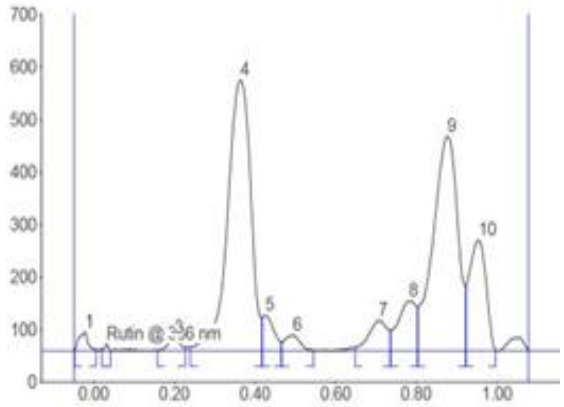
s





Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.04	1.1	-0.02	70.3	7.98	0.00	21.5	1086.6	3.54	unknown *
2	0.01	21.9	0.03	40.7	4.62	0.07	11.1	1067.3	3.48	Rutin
3	0.15	12.6	0.20	29.6	3.36	0.22	19.7	878.9	2.86	unknown *
4	0.26	23.6	0.36	554.9	62.98	0.41	91.5	22842.6	74.38	unknown *
5	0.41	91.8	0.42	97.4	11.06	0.46	30.9	2239.7	7.29	unknown *
6	0.46	31.4	0.49	52.7	5.98	0.54	8.8	1527.9	4.98	unknown *
7	0.67	11.9	0.70	17.5	1.98	0.75	2.0	577.8	1.88	unknown *
8	0.83	0.5	0.87	18.0	2.04	0.92	0.5	488.7	1.59	unknown *

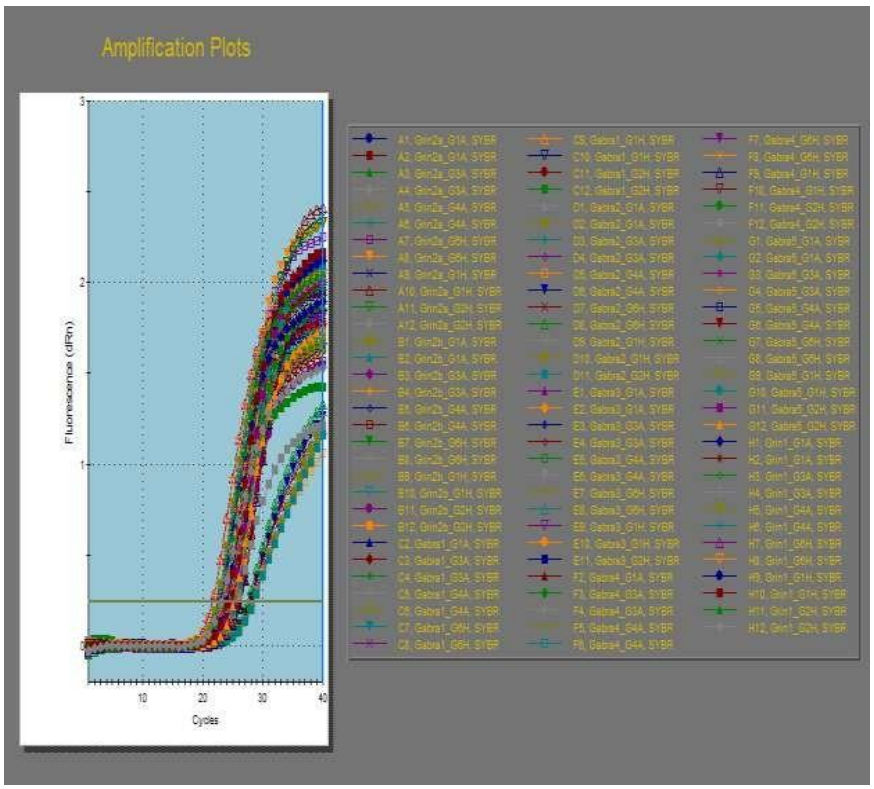


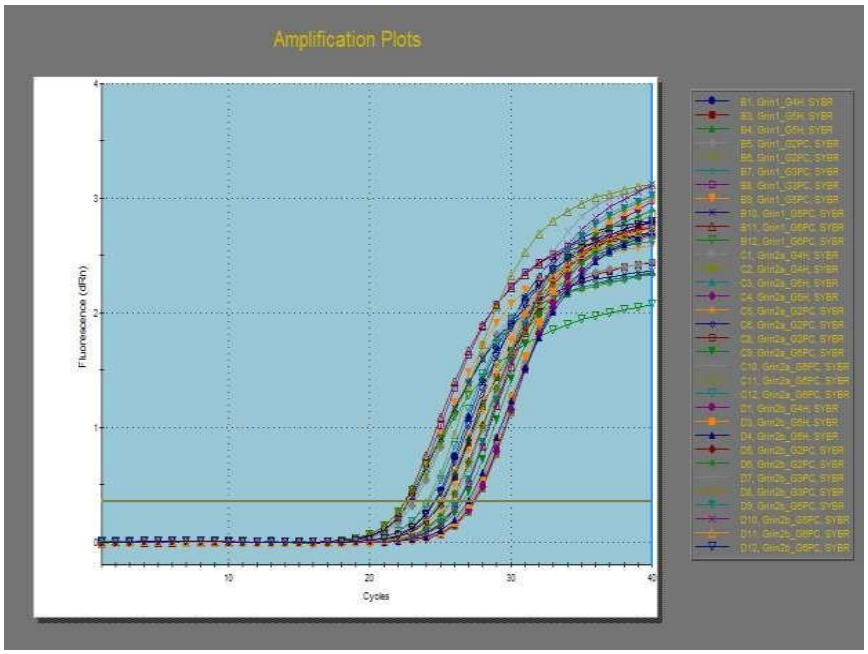


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.05	0.1	-0.02	35.4	2.43	0.01	1.7	565.0	1.05	unknown *
2	0.02	2.2	0.03	12.2	0.84	0.04	0.3	84.2	0.16	Rutin
3	0.16	1.1	0.20	23.7	1.63	0.23	6.4	499.0	0.93	unknown *
4	0.24	7.3	0.36	515.6	35.43	0.42	63.8	21168.6	39.43	unknown *
5	0.42	64.0	0.43	67.2	4.62	0.47	17.6	1384.1	2.58	unknown *
6	0.47	17.6	0.50	29.7	2.04	0.55	0.7	818.6	1.53	unknown *
7	0.65	6.2	0.71	57.4	3.94	0.74	39.1	1908.6	3.56	unknown *
8	0.74	39.2	0.78	95.1	6.53	0.80	83.4	3074.3	5.73	unknown *
9	0.81	83.9	0.88	408.2	28.05	0.92	124.2	17940.4	33.42	unknown *
10	0.93	126.0	0.96	210.8	14.49	1.00	0.8	6237.6	11.62	unknown *

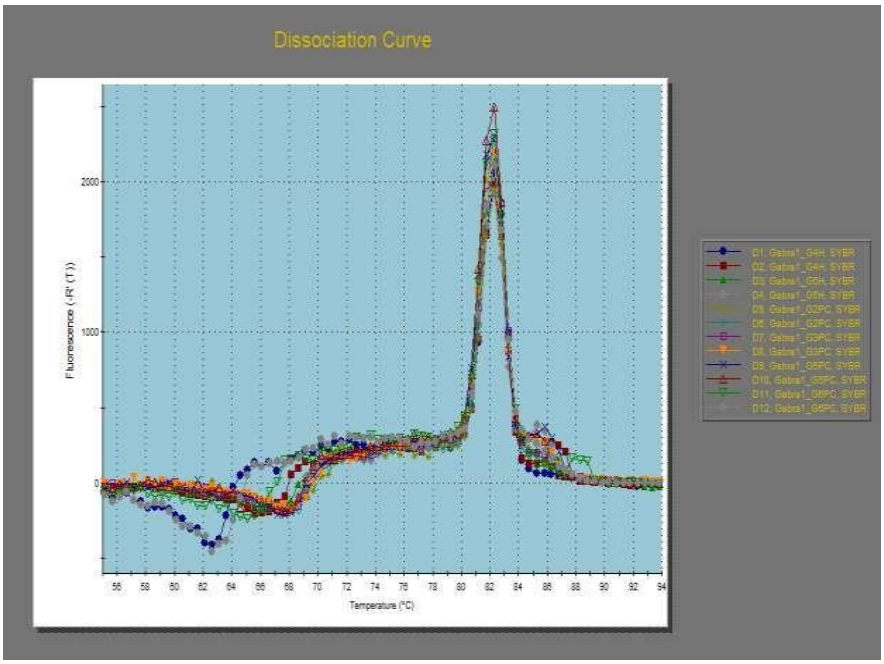
APPENDIX B

Amplification plots of all samples

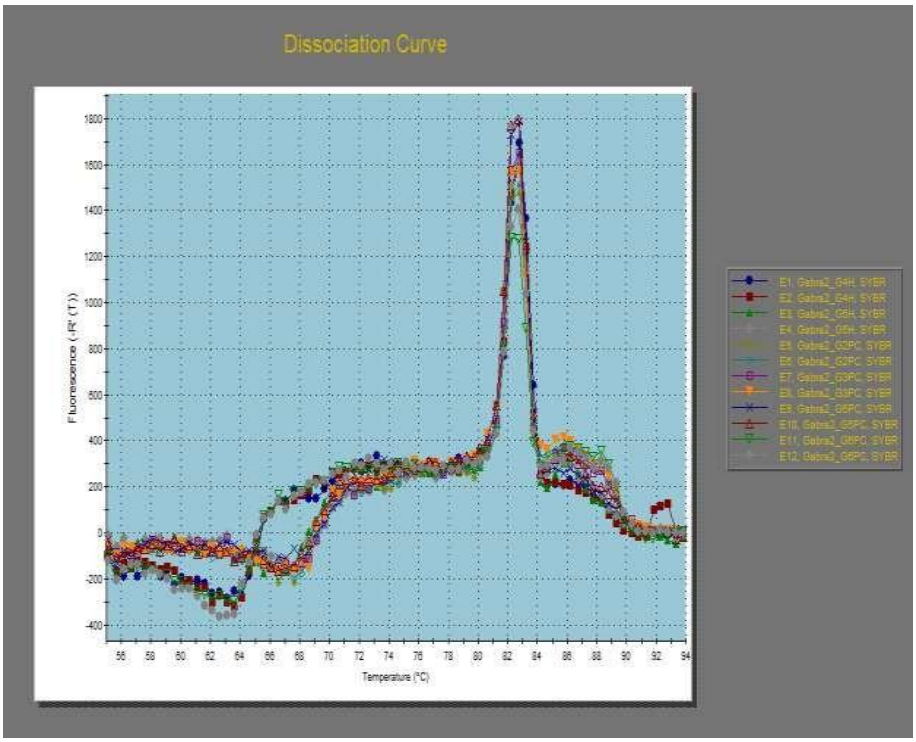




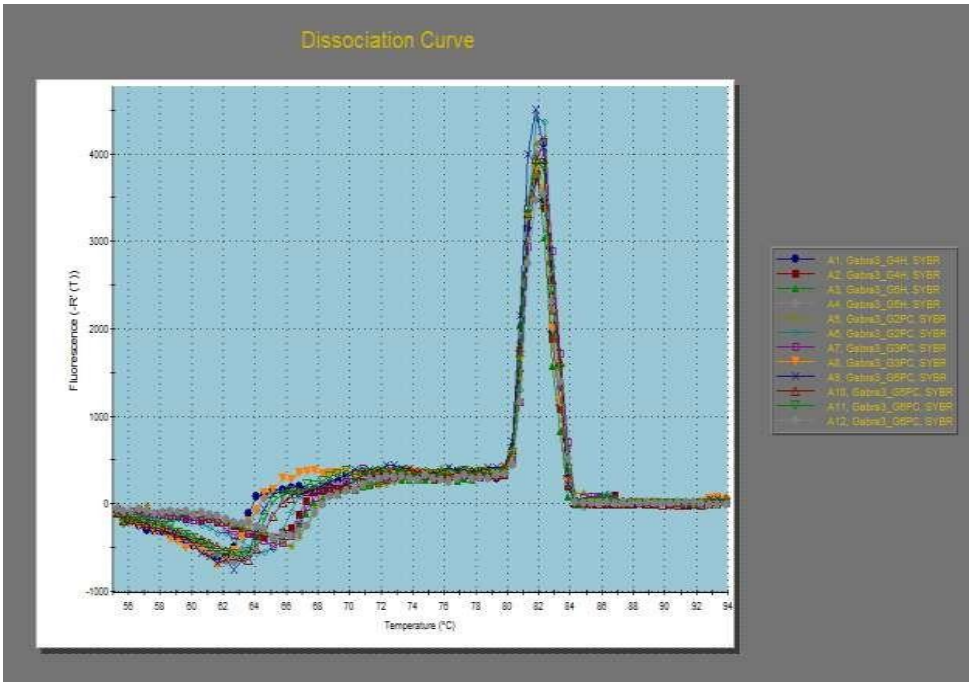
Dissociation curve for Gabra1



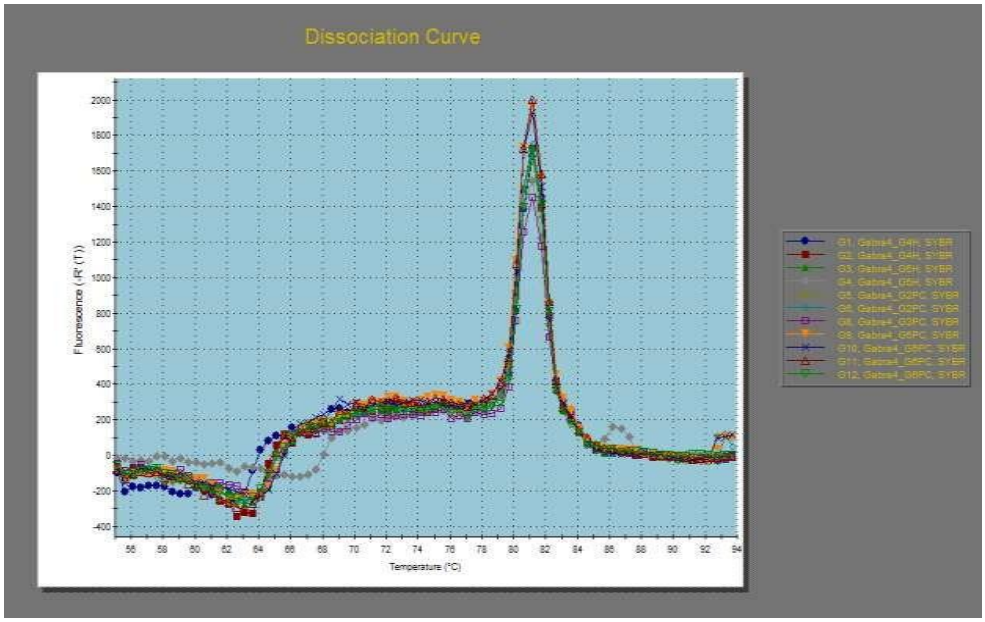
Dissociation curve for Gabra2



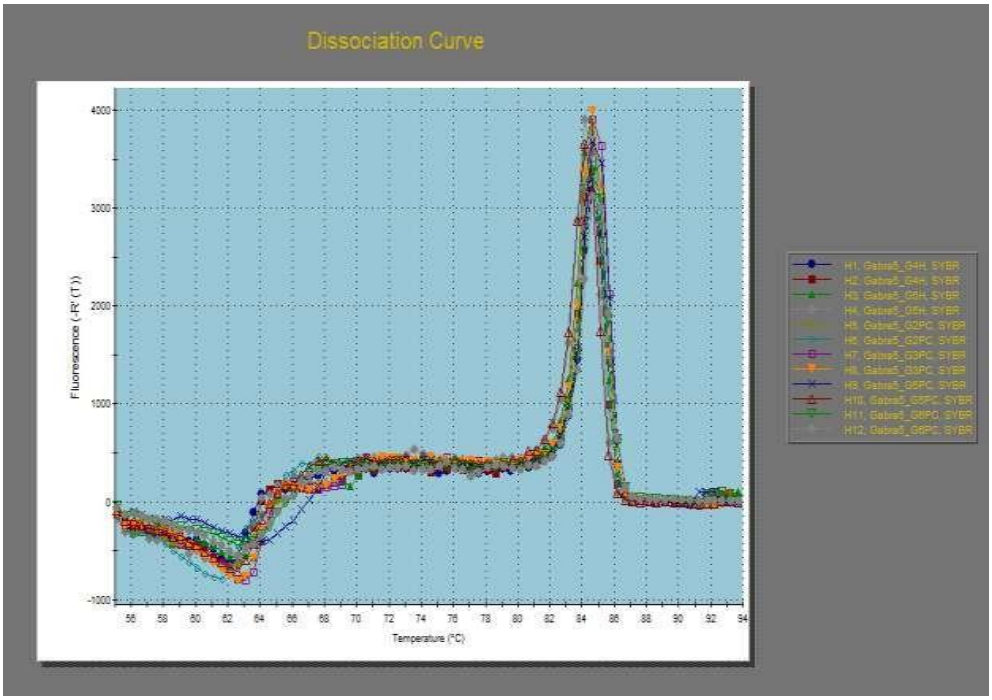
Dissociation curve for Gabra3



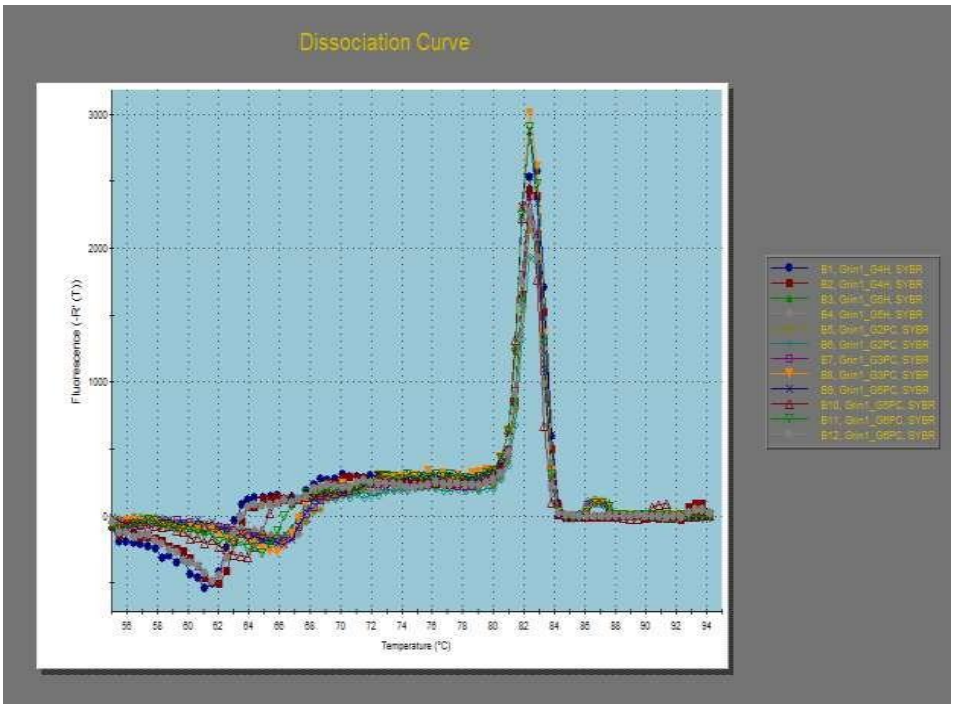
Dissociation curve for Gabra4



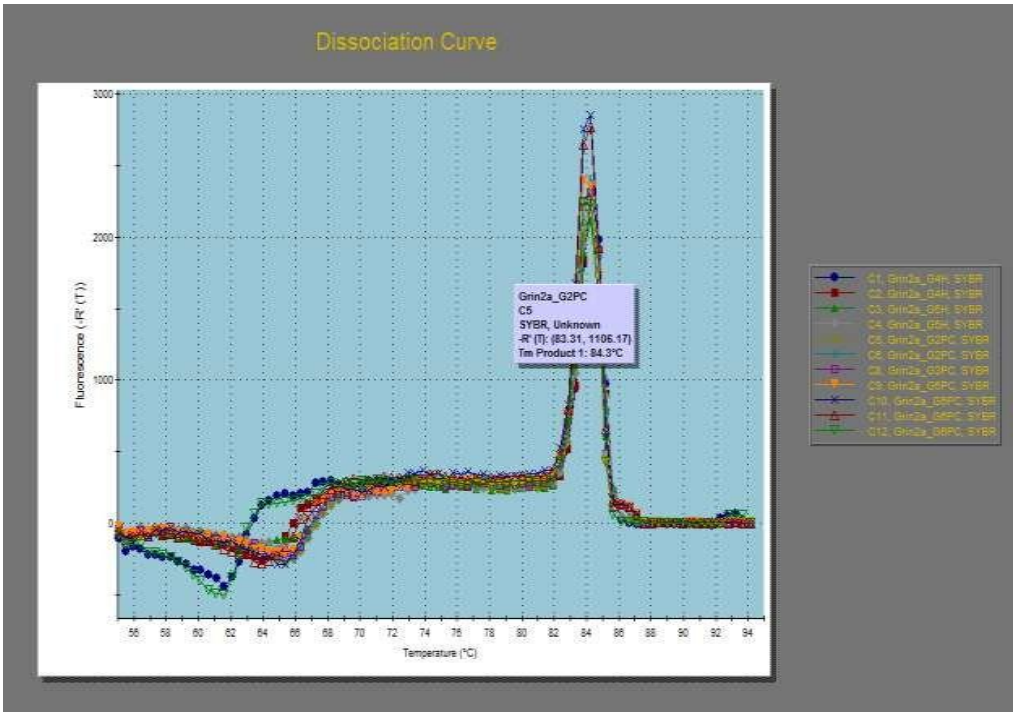
Dissociation curve for Gabra5



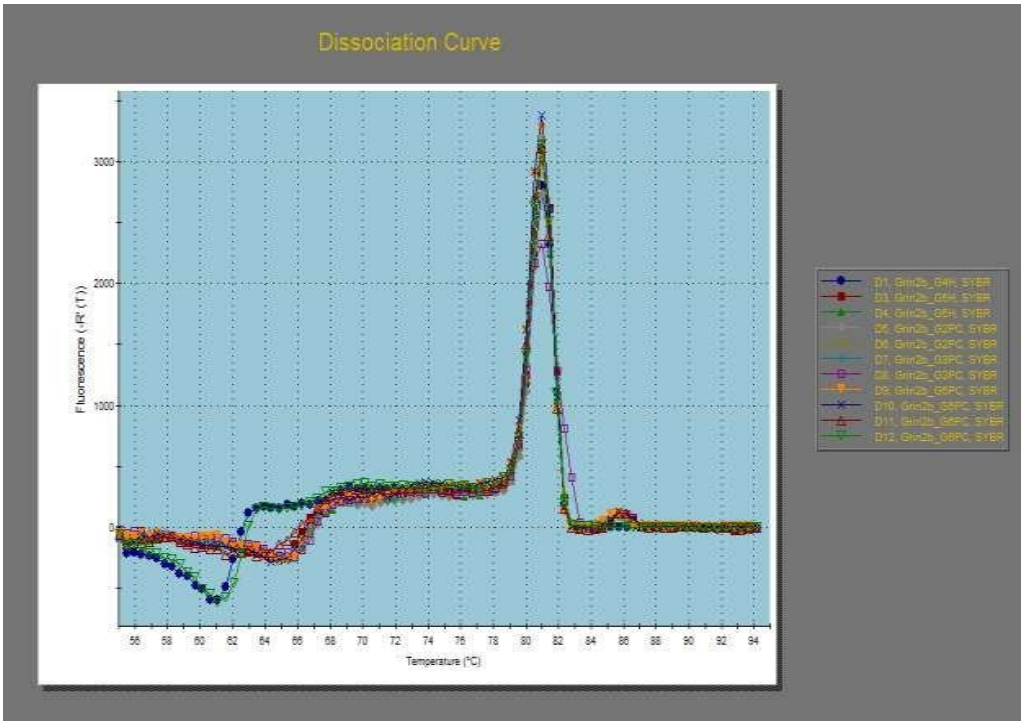
Dissociation curve for Grin1



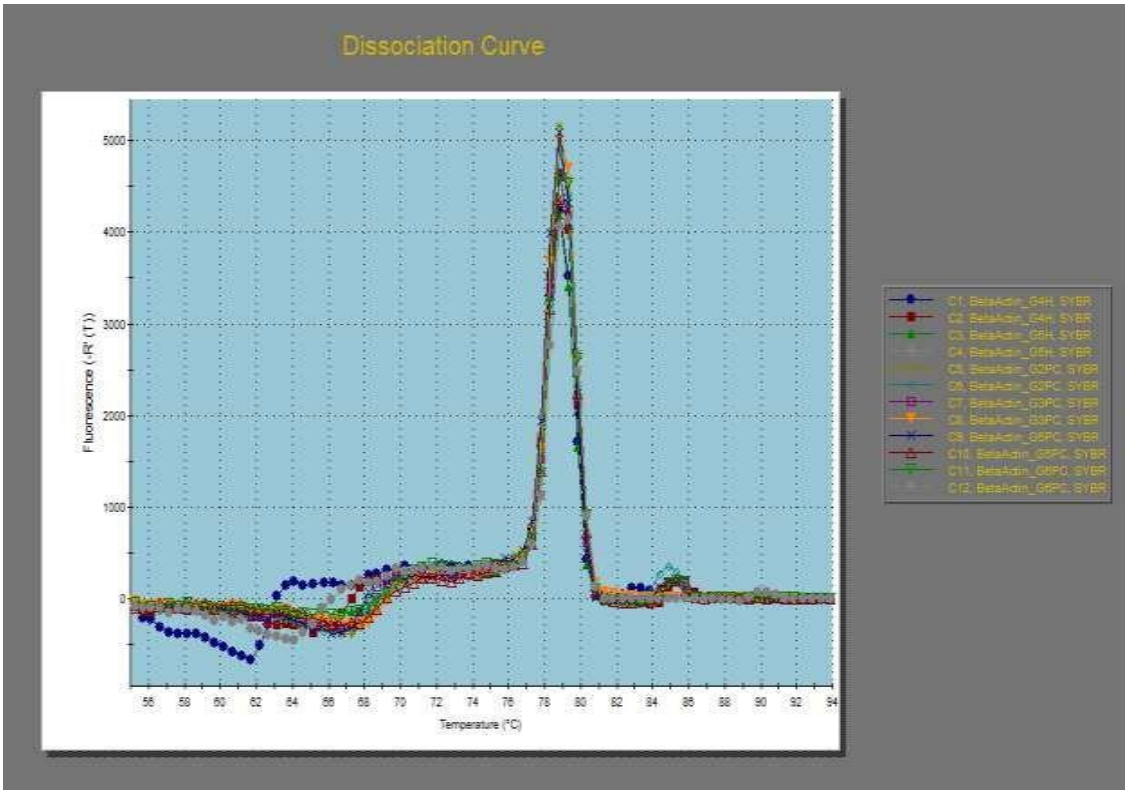
Dissociation curve for Grin2a



Dissociation curve for grin2b

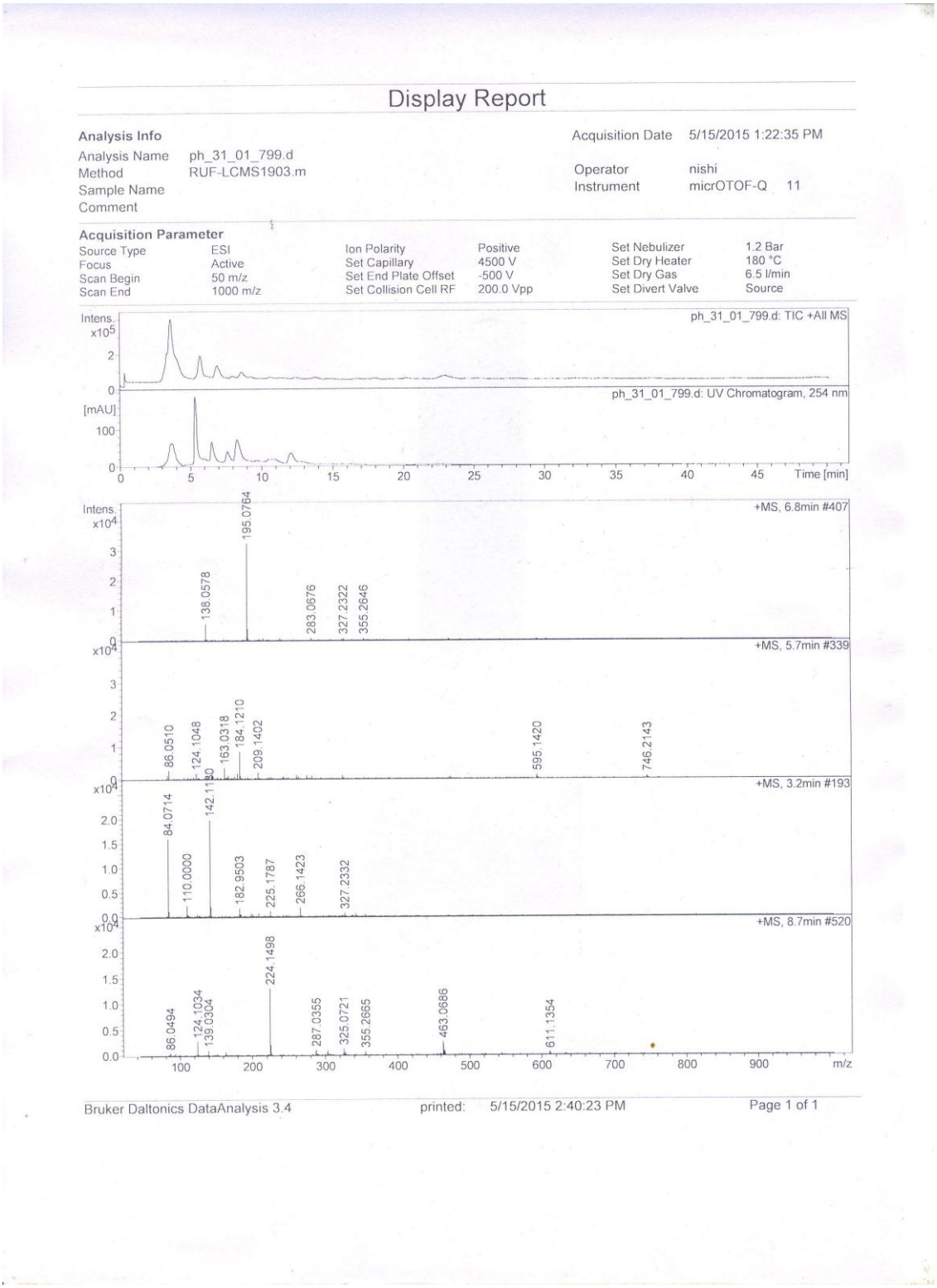


Dissociation curve for rat Beta actin



APPENDIX C

LC-MS spectrum report of POL-6



LIST OF PUBLICATIONS

1. **L. Sharma**, A. Sharma, G.L. Gupta. "Standardization of a polyherbal preparation (POL–6) for treatment of oxidative, inflammatory and immune disorders" *International Journal of Pharmacy and Pharmaceutical Sciences*. vol. 8(4), pp. 129-134, 2016.
2. **L. Sharma**, A. Sharma, G.L. Gupta, G.S. Bisht. Acute and Sub-acute. "Oral Toxicity Assessment of a Standardized Polyherbal Preparation POL-6 in Rats", *The Natural Products Journal*. vol. 9(3), pp.207-216, 2019.
3. **L. Sharma**, A. Sharma, G.L. Gupta, G.S. Bisht. "Protective effect of Ocimum sanctum Linn. leaf extract on ethanol withdrawal syndrome in Wistar rats", *Asian Pacific Journal of Tropical Medice*, vol. 11(8), pp. 423-428, 2018.
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