

**MODULATION OF TUMOR NECROSIS FACTOR
ALPHA BY PIRFENIDONE AND PIPERINE IN OPIOID
WITHDRAWAL SYNDROME**

A Thesis

Submitted in partial fulfillment of the requirements for the award of the
degree of

DOCTOR OF PHILOSOPHY

in
PHARMACOLOGY

By

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LOVELY PROFESSIONAL UNIVERSITY, PUNJAB

2023

DECLARATION

I, **Gurpreet Bawa**, hereby declare that the matter embodied in this thesis entitled "**Modulation of tumor necrosis factor alpha by pirfenidone and piperine in opioid withdrawal syndrome**" submitted to School of Pharmaceutical Sciences, Lovely Professional University, Amritsar is result of my investigations and the work and is not submitted anywhere else for the award of degree. This thesis has been composed by me under the direct supervision and guidance of **Dr. Navneet Khurana**, Professor, School of Pharmaceutical Sciences, Lovely Professional University, Phagwara, Punjab, India.

All the ideas and reference have been duly acknowledged.



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CERTIFICATE

This is to certify that the work contained in this dissertation entitled **“Modulation of tumor necrosis factor alpha by pirfenidone and piperine in opioid withdrawal syndrome”** submitted in the partial fulfillment of requirements of the degree of DOCTOR OF PHILOSOPHY in PHARMACOLOGY, embodies the original research work carried out by **Gurpreet Bawa** herself under my supervision and guidance at the School of Pharmaceutical Sciences, Lovely Professional University, Phagwara, Punjab, India.



Dr. Navneet Khurana

(Supervisor)

ABSTRACT

The focus of the present research was to evaluate modulation on TNF-alpha (α) by pirfenidone and piperine and its combination against biochemical, behavioral in precipitated, and spontaneous animal models in mice. Withdrawal syndrome also called a discontinuation syndrome is a set of symptoms occurring on discontinuation or dosage reduction of some types of medications. The opiate withdrawal syndrome emerges after repeated administration of heroin, morphine, and lasts for hours to a few days, depending upon the specific drug and the duration and dose of a prior administration. In humans, the signs and symptoms of withdrawal include stomach cramps, diarrhea, rhinorrhea, sweating, elevated heart rate, and increased blood pressure, irritability, dysphoria, hyperalgesia and insomnia. Both the precipitated and spontaneous withdrawal models are commonly used to study opiate withdrawal signs. Spontaneous opiate withdrawal occurs after the sudden discontinuation or the rapid tapering of chronically administered opiates. The withdrawal symptoms in precipitated model occurs after the use of an opioid receptor antagonist, which displaces the opioid receptor agonist. Both the models of spontaneous and precipitated withdrawal syndromes are accompanied by symptoms of withdrawal. However, the action of onset is faster in precipitated opiate withdrawal, as compared to spontaneous model. Naloxone is the most common competitive μ -opioid receptor antagonist used to precipitate opiate withdrawal. It has an extremely high affinity for μ -opioid receptors in the central nervous system. Rapid blockade of opioid receptors by naloxone often produces rapid onset of withdrawal symptoms. In the precipitated model, various opioid withdrawal syndrome has been observed, the best response was observed in the combination of pirfenidone + piperine treated group. In the case of the spontaneous model, the best response was observed in the combination of pirfenidone + piperine treated group but less in comparison to the precipitated animal model. The precipitated model was treated with morphine (5 mg/kg) for 5 days via i.p administration. On sixth day, naloxone (8 mg/kg, i.p.) was administered after 2 hr of morphine administration to precipitate the opioid withdrawal symptoms. In spontaneous animal model, morphine was administered (5 mg/kg, i.p.) two times a day for consecutive 5 days. After these five days, normal saline (5 ml/kg, i.p.) was

administered instead of naloxone. Behavioral observations were done for 30 min immediately after the last injection to check the spontaneous and precipitated behavioral response of animals. Biochemical changes in the hippocampus of midbrain regions of mice brain were carried out by estimating the levels of oxidative stress markers such as thiobarbituric acid reactive substances (TBARS), glutathione (GSH), catalase (CAT), and superoxide anion generation (SAG), and TNF- α . Behavioral alterations were assessed by evaluation of jumping frequency, circling frequency, forepaw licking frequency, rearing frequency, withdrawal severity score, locomotor activity, analgesic effect, anxiety effect, depressant effect, urination frequency, defecation frequency. Neurochemical changes were assessed by estimating levels of TNF- α hippocampus of the midbrain regions of mice brain. Pirfenidone was administered at low and high doses i.e. 200 and 300 mg/kg; p.o. in both precipitated and spontaneous animal models, respectively. Piperine was also administered at two dose levels, low (10 mg/kg; p.o.) and high (15 mg/kg; p.o.) to mice of both groups. Pirfenidone and piperine were evaluated for their synergistic effect in the present study and the mice of the combination group were treated with pirfenidone + piperine (200 mg/kg+10 mg/kg respectively; p.o.). All treatments were given once a day for a consecutive period of 5 days. Comparison of all treated groups was done with opioid withdrawal group in both the models of study and also with standard and memantine treated groups for the various evaluations. After chronic administration of morphine twice daily for a period of 5 days, naloxone was given on 6th day after the two hr of morphine administered in the precipitated animal model. Cessation of morphine on 6th day in spontaneous animal model was used to induce opioid withdrawal syndrome like jumping frequency, circling frequency, forepaw licking frequency, rearing frequency, withdrawal severity score, locomotor activity, analgesic effect, anxiety effect, depressant effect, urination frequency, defecation were significantly reversed by co-administration of pirfenidone + piperine. The increase in TBARS, SAG, and decrease in GSH and CAT induced by morphine withdrawal syndrome were significantly reversed by pirfenidone and piperine co-administration. Withdrawal symptoms of morphine increased the level of TNF- α in the brain which was attenuated by pirfenidone and piperine co administration. In all evaluations, such as behavioral, biochemical, evaluations, both pirfenidone and piperine showed

significant attenuating effects at both doses but effects of combination of pirfenidone and piperine were better than the individual drug. This in turn was observed to be better than standard drug. All the above-discussed effects of pirfenidone, piperine, and their combination treatment were found to be better in the modulation of TNF- α in an opioid withdrawal syndrome.

Keywords: Opioid withdrawal, pirfenidone, piperine, morphine, naloxone, TNF- α .

ACKNOWLEDGEMENT

This golden hour fills me with immense pleasure and satisfaction as I complete the most important assignment of my life lived till date. And, as I look back on the journey that has made me embark on this destination, my heart is full of gratitude & honor towards individuals without whom I find it difficult to imagine the accomplishment. At the outset, with hands folded and head bowed down, I thank the omnipotent, the omniscient, the “Ek Onkar” Almighty for keeping me blessed with his spiritual support all long.

*I wish word had the power to express my gratitude and homage towards my very esteemed and reverend guide **Dr. Navneet Khurana** whose expert supervision, patience, support, attention to minutest details and the “be the labour great or small, do it well or not at all” attitude provided me the courage, confidence and dedication that was needed for this endeavor. Like a caring, preserving and vigilant guardian he has always been beside me through all waters-calm and rough, to ensure that I sailed safe across the voyage. His clear understanding of subject and open -minded discussions has helped me expand the horizons of my thinking and understanding of the subject to newer skies.*

*I sincerely thank to **Mr. Ashok Mittal**, Chancellor, Lovely Professional University, Punjab, **Mrs. Rashmi Mittal**, Pro-Chancellor and **Dr. Monica Gulati**, Senior Dean, Lovely Faculty of Applied Medical Sciences for providing all the necessary infrastructural facilities as well as excellent working environment in the laboratory in order to complete this task. I am very thankful to **Dr. Rekha** (Assistant Dean, Centre for Research Degree Program, LPU) for her motivational support during the course.*

*I deeply thank to **Dr. Bimlesh Kumar** (In-charge animal house) and the outstanding faculty members of the school of pharmaceutical Sciences, namely **Dr. Amit Mittal, Dr. Bhupinder Kapoor, Dr. Sheetu Wadhwa, Dr. Sazal Patyar, Ms. Neha Sharma, Mr. Dileep Singh Bhagel, Dr. Sachin Kumar Singh and Mr. Gopal Krishan Duvedi** for providing indispensable advice, information and timely assistance indifferent aspects during mywork.*

*I am extremely thankful to the non-teaching staff namely **Mr. Madan, Mr. Vinod and Mr. Raju** for their generous support and cooperation. I pray my special thanks to all the rats who sacrificed their lives for this research work.*

*I thank deeply from my heart to my beloved parents **Mr. Yashpal Singh Bawa & Late Mrs. Narinderjit Bawa** for their blessings, motivation and support. They have always trusted their insights in me. I am very thankful to my parents in law **Mr. Suman Kumar Bansal & Mrs. Neelam Bansal** for their blessings and cooperation. I am lucky to be blessed with the most caring and encouraging parents.*

*I am especially thankful to my dear and beloved husband **Mr. Mohit Bansal** for always helping me to get through the difficult times and for all emotional support & entertainment that he provided. I also thank my Mama Ji **Mr. Vikas Gupta**, brother **Mr. Tarun Aggarwal, Mr. Jaspreet Singh Bawa** & sister in law **Ms. Jaspreet Kaur Bawa** for their emotional support and rendering all sorts of help. I feel immense joy to mention the names of my beloved daughter **Gloshini Bansal** and my beloved nephew **Virat Singh Bawa** whose cute smiles help me to face obstacles smoothly & fill me with enchant.*

*I also want to give special thanks to **M/S Merck India Limited** for providing the test drugs for my research work without it would be difficult for me to start the practical work*

*I also extend my gratitude to drug inspectors **Mr. Navdeep Singh and Mr. Karun Kumar** for extending their support for getting the permission of morphine usage in my research work.*

*I am very thankful to the research scholars **Mr. Rajan Kumar & Mr. Rakesh** and my colleagues **Mr. Rajan Salwan, Mr. Mannan Khanna and Ms. Priyanaka** for their help and cooperation.*

A warm and sincere thanks to all my family members, teachers and friends.

A handwritten signature in blue ink that reads "Gurpreet Bawa". The signature is written in a cursive style with a horizontal line underneath the name.

Gurpreet Bawa

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LIST OF ABBREVIATIONS

- 5HT:** 5-hydroxyl Tryptamine
- AAT:** Alanine Amino Transferase
- AsAT:** Aspartate Amino Transferase
- AChE:** Acetylcholine-esterase
- AC:** Adenylyl Cyclase
- AMPK:** Adenosine monophosphate activated protein kinase
- BBB:** Blood brain barrier
- TLR:** Toll Like Receptor
- CRF:** Corticotrophin-releasing Factor Signaling
- CNS:** Central nervous system
- COX2:** Cyclooxygenase-2
- COX:** Cyclooxygenase
- CVS:** Cardiovascular system
- CXCL:** Chemokine ligand
- CYP:** Cytochrome P
- DA:** Dopamine
- DNA:** Deoxyribonucleic acid
- DLC:** DORSAL longitudinal columns
- DRN:** Dorsal Raphe Nuclei
- ER:** Endoplasmic reticulum
- ERK:** Extracellular-signal-regulated kinase

ETC: Electron transport chain

FADD: Fas-associated death domain

Fe²⁺: Ferrous ion

Ig: Immunoglobulin

IGR: ionotropic Glutamate receptors

IκB-α: kappa B-alpha

IL-6: Interleukin-6

IL-1β: Interleukin-1beta

iNOS: Inducible nitric oxidase synthase

JNK: c-Jun NH2-terminal kinase

KOR: kappa opioid receptor

LDL: Low-density lipoprotein

LN: Lewy neuritis

LPS: Lipopolysaccharide

MAP: Mitogen-activated protein

MAPKs: Mitogen-activated protein kinases

MHC: Major histocompatibility complex

MMP: Matrix metalloproteinase

MPDP: 1-methyl-4-phenyl-pyridinium ion

MPP⁺: Pyridinium ion

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

Na⁺/K⁺ ATPase: Sodium potassium ATPase

NADPH oxidase: Nicotinamide adenine dinucleotide phosphate oxidase

NF-κB: Nuclear factor-kappa

NGF: Nerve growth factor

NIK/IKK: Nuclear factor-inducing kinase/I κ B kinase

NLRP3: Nucleotide-binding domain and leucine-rich repeat containing proteins

NMDA: N-methyl-D-aspartate

NO: Nitric oxide

NOAEL: No observed adverse Effect level

NOX: NADPH oxidase

O^{2•-}: Superoxide anion

OH•: Hydroxyl radical

PARP-1: Poly-(ADP-ribose) polymerase

PCR: Polymerase chain reaction

PD: Parkinson's disease

PGR: Periaqueductal Gray Region

PPAR: Peroxisomal proliferator-activated receptor

PTP: Permeability transition pore

PNC: primary neurilemma cells

ROS: Reactive oxygen species **SAA:** Sulfur amino acids

SAG: Superoxide anion generation

SAH: S-adenosylhomocysteine

SAM: S-adenosylmethionine

SEM: Standard error of mean

SN: Substantia nigra

SNc: Substantia nigra *pars compacta*

SOD: Superoxide dismutase

SSMG: single-spanning membrane glycoprotein's

TBARS: Thiobarbituric acid reactive substances

TDP: Transactive response DNA binding protein

TIMP: Tissue inhibitor of metalloproteinase

TLR: Toll-like receptors

TNFR: TNF receptor

TNF- α : Tumor necrosis factor-alpha

TST: Tail suspension test

UCHL: Ubiquitin carboxy terminal hydrolase

VTR: ventral tegmental region

VLC: ventrolateral longitudinal columns

VPGR: ventrolateral periaqueductal gray region

VLM: ventrolateral medullary

1. INTRODUCTION

Opioids take condemnatory contribution in accomplished pain consolation in both current and prehistoric pharmaceutical formulations. But, their clinical utilization could be inadequate, secondary to undesirable and unfavorable effects such as toleration, helplessness, reward, and alteration in behavior. People who are suffering from opioid withdrawal syndrome are most likely three categories, first the younger adults (15 to 25 yrs), second are those who are suffering from mental disorders like depression, anxiety, post traumatic stress disorder and third category are those of persons who have history or past of drug abuse. Opioid dependence emerges after its long-term utilization. If it is left untreated, high morbidity and mortality rates are seen (Cruts *et al.*, 2008; Clausen *et al.*, 2009). Psychosocially assisted pharmacological treatment of opiate dependence is used to reduce illicit opiate abuse, reduce the harms related to opiate abuse and improve quality of life (Amato *et al.*, 2008; WHO, 2009). Withdrawal syndrome, also called a discontinuation syndrome is a set of symptoms occurring on discontinuation or dosage reduction of some types of medications. The opiate withdrawal syndrome emerges after repeated administration of heroin, morphine, and lasts for hours to a few days, depending upon the specific drug and the duration and dose of prior administration. In humans, the signs and symptoms of withdrawal include stomach cramps, diarrhea, rhinorrhea, sweating, elevated heart rate and increased blood pressure, irritability, dysphoria, hyperalgesia, and insomnia (Morgan and Christie 2011).

Animal models of acute and chronic exposures were found to be useful in understanding opiate tolerance and dependence (De Vries and Shippenberg 2002; Harris and Gewirtz 2005). Acute opiate exposure has been defined as the administration of an opiate in single or multiple dosages over 1 to 2 days, whereas chronic opiate exposure has been defined as administration of an opiate for a minimum of 5 days (Georges *et al.*, 2000; Laorden *et al.*, 2002). In the preclinical setting, the opiate withdrawal syndrome is well characterized in rodents. Both the precipitated and spontaneous withdrawal models are commonly used to study opiate withdrawal signs. Spontaneous opiate withdrawal occurs after the sudden discontinuation or the rapid tapering of chronically administered opiates. Precipitated

opiate withdrawal occurs after the use of an opioid receptor antagonist, which displaces the opioid receptor agonist. Both spontaneous and precipitated withdrawal syndromes are accompanied by symptoms of abstinence; however, the onset is faster with precipitated opiate withdrawal. Naloxone and Naltrexone are the most common competitive μ -opioid receptor antagonists used to precipitate opiate withdrawal (Reti and Baraban 2003). Naloxone is a drug used to counter the effects of opiate overdose, e.g. heroin or morphine overdose. It has an extremely high affinity for μ opioid receptors in the central nervous system. Its rapid blockade of those receptors often produces rapid onset of withdrawal symptoms.

A substantial investigation has preceded the enlargement, nonperpetual opioids, and agents that can countermand and intercept the addiction method for example methadone, a chronic opiate, which can also be utilized for removing the toxins from the body by opiate drugs. Efficient in diminishing the signs of narcotic disengagement extremely for parenteral narcotic patients. Diazepam plays a major role at the GABAA receptor and is utilized to investigate anxiety, persistent problems falling and staying asleep, soreness in the muscle that ranges from mild to severe, and cravings. A non-opiate alpha-2 agonist i.e. clonidine lessens sympathetic discharge to the body. Clonidine, a non-opiate alpha-2 agonist, decreases sympathetic outflow to the body. This can often reduce the symptoms of opiate withdrawal. Benzodiazepines, such as diazepam, work at the GABA 2_A receptor and are used to treat agitation, insomnia, muscle aches, and cravings. However, none of the available options promises to conclusively treat the condition of opioid dependence and its related abstinence syndrome (Law *et al.*, 2000).

Opiate drugs exert their effects by binding to three opioid receptor types (μ , γ , and κ) and mimicking the actions of endogenous opioid peptides, the endorphins, endomorphins, enkephalins, and dynorphins. The μ opioid receptor (MOR) subtype is critical for the rewarding effects of heroin and morphine. The most prominent neuroadaptive changes during morphine induced dependence include desensitization of MORs and up regulation of the cAMP pathway. The mitogen activated protein kinase (MAPK) pathway as well as Ca^{2+} signaling are also affected during morphine dependence. The primary consequence of morphine withdrawal is „super activation“

of adenylyl cyclase (AC) and a subsequent overproduction of its downstream signaling molecule, cAMP. Other cAMP actions during withdrawal include PKA-mediated enhanced GABAergic synaptic transmission in areas such as periaqueductal grey (PAG), ventral tegmental area (VTA), nucleus accumbens (NAcc.) and dorsal raphe (Williams *et al.*, 2001; Bailey and Connor 2005). Among the brain regions implicated in opiate dependence and withdrawal, the periaqueductal gray area (PAG) appears to be critical in regulating the complex signs and symptoms of opioid withdrawal. Numerous neurochemical mechanisms in the PAG have been identified that may contribute to the opioid withdrawal syndrome (Handong Ouyang *et al.*, 2012). Other receptors like glutamate, muscarinic, nicotinic and toll like receptors are also involved in morphine withdrawal syndrome.

Toll like receptors play important role in morphine withdrawal syndrome in which glial cells, particularly astrocytes, envelop neuronal synapses and participate in the physiological control of synaptic transmission and plasticity via the release of synaptically effective mediators, a process called gliotransmission (Halassa *et al.*, 2007; Haydon *et al.*, 2009) Morphine binds to an accessory protein of glial toll-like receptor 4 (TLR4), myeloid differentiation protein 2 (MD-2), thereby inducing TLR4 oligomerization and triggering proinflammatory cytokines (Wang *et al.*, 2012). Direct activation of glial TLR4 induces overexpression of TNF- α (Bettoni *et al.*, 2008; Saito *et al.*, 2010). Morphine withdrawal induces astrocytic activation to release TNF- α in the PAG. It has also been proved that exogenous TNF- α injection into the PAG evokes morphine withdrawal-like behaviors (Hao *et al.*, 2011).

Piperine has various medicinal uses. It has antidepressant, bioenhancer, apoptosis inhibitor, genotoxicity, antioxidant, antiplatelet, hepatoprotective, antithyroid, anti-inflammatory, antihypertensive, fertility enhancer antitumor and antiasthmatic activity. In different examinations of piperine that threaten the adherence of WBC to endothelial first layer because of blockage of tumor necrosis factor- α presented the cell bond particles for example intercellular attachment atom 1, vascular cell grip particle 1, and E-choosing (Sarvesh *et al.*, 2007). It is seen in pre-finding of endothelial cells with piperine that the obstruction just as phosphorylation of I κ B α is curbed by contracting cancer rot factor- α affected I κ B kinase pursuit.

Exacerbation of the collagen system assault of B16F-10 melanoma cells in a portion subordinate way was seen with piperine at various fixations at 2.5, 5, and 10 µg/milliliters. The favorable to provocative cytokines like IL-1β, IL - 6, GM-CSF and Tumor Necrosis Factor - alpha were noticeably diminished (Pradeep *et al.*, 2004). The rationale of the study is to see the effect of test drugs in decreasing the level of TNF-α in mice brain for treating the opioid withdrawal syndrome.

2. REVIEW OF LITERATURE

2.1 Opioid Withdrawal Syndrome

The Opioid Withdrawal Syndrome (OWS) attracted the interest of the society and the scientists since the realization in 1880's which the useful opiate drugs are induced in the state of the dependency of psychological and physiological relationships (McPhail et al., 2021). The explanations for the syndrome of the disturbance of the autonomic and the distress of the psychic that appears when the intake of the drug ceases. That is ranged to elaborate the moral theories and psychological to increase the precise of the biochemical theories. The new compounds that are introduced partly by the lack of the effects of withdrawal that have induced in both methadone and heroin. The withdrawal syndromes and for the substantial abuse. The treatment suspected to affect the function of the brain that has tried without the important success against the withdrawal syndrome. It is clearly discussed below on various existing studies that concentrates on the effects of the OWS that affects the brain function to the adults and even affects the infants from the mother who has OUD.

In 2015, it was found that approximately 800,000 subjects used morphine, while 4 million misused prescription opioids. Although use of other drugs such as heroine and cannabis is more prevalent, opioid use contributes to significant morbidity, mortality, and social and economic costs. While the current US opioid overdose epidemic began with prescription opioids, since 2015, heroin and synthetic opioids (e.g., fentanyl) have driven continued increases in opioid overdose deaths, contributing to a recent decline in overall life expectancy in the United States. Policies to address the opioid epidemic by changing clinical practice include provider education, monitoring prescribing practices, and expanding the clinical workforce necessary to treat opioid use disorders. The opioid epidemic appears to be largely a US phenomenon and a consequence of both structural challenges in the US healthcare system and growing socioeconomic disparities, and thus it will require policies including and beyond delivery system reforms to resolve it.

This syndrome is a condition that is life threatening and that results from the base of dependence in opioid. The opioids are set of drugs that is used for managing severe pain. These substances are commonly used as the psychoactive all around the earth. These includes drugs such as heroine, codeine, morphine, methadone, hydromorphone hydrochloride, and oxycontin. Using these drugs produce pain relief, euphoric feelings and mental relaxation. Using these chronic opioid leads to the form of dependence that incapacitating to a

development in users. This dependence create an impact on the users of drug and that imposes an important economic burden inside the society by increasing the rate of unemployment's, premature mortality, cost of health care and absenteeism.

Animal models of “acute” (i.e., a single use) and “chronic” (i.e., long-term use) exposures have been useful in understanding opiate tolerance and dependence (De Vries and Shippenberg 2002; Harris and Gewirtz 2005). Acute opiate exposure has been defined as the administration of an opiate in single or multiple dosages over 1 to 2 days, whereas chronic opiate exposure has been defined as administration of an opiate for a minimum of 5 days (Georges *et al.*, 2000; Laorden *et al.*, 2002). In the preclinical setting the opiate withdrawal syndrome is well characterized in rodents. Both the precipitated and spontaneous withdrawal model are commonly used to study opiate withdrawal signs. Spontaneous opiate withdrawal occurs after the sudden discontinuation or the rapid tapering of chronically administered opiates. Precipitated opiate withdrawal occurs after the use of an opioid receptor antagonist, which displaces the opioid receptor agonist. Both spontaneous and precipitated withdrawal syndromes are accompanied by symptoms of abstinence; however, the onset is faster with precipitated opiate withdrawal. Naloxone and Naltrexone are the most common competitive μ -opioid receptor antagonists used to precipitate opiate withdrawal (Reti and Baraban 2003). Naloxone is a drug used to counter the effects of opiate overdose, for example heroin or morphine overdose. It has an extremely high affinity for μ -opioid receptors in the central nervous system. Its rapid blockade of those receptors often produces rapid onset of withdrawal symptoms.

OWS occurs when the patient who was dependent in using drugs suddenly reduces or if the person stops taking opioids. This can also cause to the patients who has opioid in their system and that given a partial agonist opioids like antagonists or buprenorphine and like naltrexone or naloxone. The opioid withdrawal etiology is complex. Various studies have shown that from the animal models indicated the opioid withdrawal symptoms are closely related to the paths based on the central excitation that is adenylyl cyclase superactivation.

Opioid use disorder management becomes challenging as crisis of the opioid intensifies increasingly, that review discusses about the physiology of the opioid receptor and pathophysiology and the symptomatology of the opioid withdrawal as well the current treatment options that are available in withdrawal position to assist the individuals with the Opioid use disorder and the physical dependence (Dydyk, Jain, and Gupta, 2021). The existing study conducted a database of the PubMed to synthesize the present understanding of

the Opioid Use Disorder and the symptoms of the Opioid withdrawal. This gives the background for managing the use of opioid and the treatment. Totally 14 researches were conducted using the term ‘Opioid Withdrawal Symptoms’ and the ‘Opioid withdrawal neurophysiology’ that unfiltered by the date. After filtering the irrelevant results and the redundancies, then One hundred and sixteen articles were considered for the process of evaluation. Many opioid based medicines are available to allay the OWS, in the period of acute phase of detoxification, supporting with the evidence of the pharmacotherapy use combining with the support of the short and long term psychosocial management. Drug based medication faces many challenges for so many reasons that includes the shortage of the requirement of specific listening or the qualified doctors, and lack of the training process and giving the support to the doctors who shows interest in treating the patients with the Opioid Use Disorder. There is also an on going want for some additional treatments that are effective considering those as options to minimize the OWS. Many patients who used non-opioid drugs also included in the target of getting the symptoms automatically that are connected to the hyperactivity of the noradrenergic, this serves a significant supportive role in delivering the system of the comprehensive detoxification of opioids (Pergolizzi Jr, Raffa, and Rosenblatt, 2020).

The important objective of the existing study are to evaluate the development of the utility of the morphine dose that responses to the model by the clinical response of Bayesian Forecasting, and to develop and check the MFS relationship that uses to collect the routine clinical data from the electronic medical records of the infants who have NOWS for adjusting the infant and maternal base factors. The existing study collected the data by using the cohort retrospective design, by reviewing the medical records of the infants who are admitted to the Neonatal Intensive Care Unit (NICU) level IV of University of Maryland Medical Centre in between 2013th January to 2017th December (Carter, Mulder, Bartram, and Darlow, 2005). Using diagnostic codes the retrospective codes were identified, and from the electronic medical charts and records from the hospitals shows drug withdrawal, Neonatal Abstinence Syndrome and drug exposure. NOWS optimizes the morphine dosing and the treatment a priority, from the given unknown effects of the early exposure to the opioid on the infants and development of the child and given from the nationwide epidemic of Opioid the steps to make the changes were taken. The reports of the infants suggested that they are exposed to the prenatal opioids that makes them to born small of the gestational age, the infants are born

with smaller heads and it is reduced in the neurocognitive performance of in their childhood. (Wijekoon, Aduroja, Biggs, El-Metwally, and Gopalakrishnan, 2021)

The main purpose of the existing study is to determine the detoxification length that is more affective when the withdrawal symptoms is reducing. The Opioid detoxification process is in effect with the toxic substances to excrete all those from the body. This becomes the adequate treatment for detoxification for the use of the Opioid Use Disorder is the essential process. The length of the stay as well the pharmacological use of the detoxification process are the most significant aspects in the treatment. A patient who is adequately treated during the process of detoxification will lead to the better outcome of the treatment. The existing study compared to the scores of the Clinical Opioid Withdrawal Scale COWS in the beginning and in the end of the detoxification process with the use of the Subutex, in the time period from five to seven days of stay (A. Srivastava, Kahan, Njoroge, and Sommer, 2019). The evaluated data is compared using the paired t-tests and the independent tests. The existing study took place at Northern New Jersey in the facility of 16- beds in the process of detoxification. The COWS is the tool that is commonly used in the process of detoxification to assess the symptoms after the withdrawal when it is treated with the Subutex.

The results in the existing study suggests that the patient who detoxes from the opioids will do better after the completion of the seven day detoxification process. Based on the results of the present study the facility should collect the additional data's to access the patients who have the relapse rate to stay for five days length and also about the patients who got discharged due to the relapse of opioid. (Melendez, 2020)

The aim of the existing study is to compare the fentanyl which is routinely administered by using drug in the ICU's to control pain and withdrawal. But with the methadone it is widely accepted as the medication that can be treated to the withdrawal syndrome opioid dependence patients who are admitted in hospitals. The addicted patients who intubated their symptoms and signs of the withdrawal that initiated during the stay in the ICU are also included. The severity of the symptoms of Withdrawal is considered as criteria of inclusion which are moderate, severe symptoms of withdrawal and mild. Patients who has other dependencies are excluded.

Later the patients assigned to the separate groups alternately to fentanyl or methadone groups. In the Fentanyl group the patients were intravenously administrated of fentanyl with initial dosage of 50 to 100 µg per hour that is adjusted subsequently based on the response from the

patients. If a patient received 50 to 100 µg per hour then the mean dose of 75 µg per hour is used for the calculations and it is also considered for the purpose of analysis. Later the patients are asked to re-visit and then their Clinical Opiate Withdrawal Score is also re-calculated. In Methadone group the patients were put on the subcutaneous methadone by giving the initial dose of 10 mg for every 12 hours that means 20 mg every day in the beginning and later the dosage were adjusted subsequently based on patients who have withdrawal symptoms and signs. Then the concluded with the dosage of 0.1 mg for every 8 hours and adjusted to a maximum rate of dosage as 1.2 mg. The evaluated outcome was the alleviation of the withdrawal symptoms and signs. The secondary outcomes are the withdrawal syndrome's duration and the duration that the patient stayed in the ICU and in the hospital, the intubation duration and the development of the later symptoms and signs and the later developed complications that are aspiration pneumonia, Acute Respiratory Distress Syndrome (ARDS), Acute Tubular Necrosis, Rhabdomyolysis, and the bed sores all that prolonged due to the intubation. Also required the further sedatives to treat the agitation. The Addiction of Opioid is confirmed by the history of the patients and by the urine tests which are positive and the initiation of the Clinical Opiate Withdrawal syndrome that determines by using the COWS. By evaluating the data's that are recorded and passed to the Statistical package for the social sciences SPSS. Between the two groups the intubation duration, hospital stay and the stay in the ICU are similar. The only important factor that is different between two groups are the alleviation of the withdrawal symptoms and signs after the medication administration need and which is importantly shorter in the group of methadone. (Najafi et al., 2021)

The crisis of the Opioid has grown to affect the pregnant women and the infants across the US, as the evidence by the rates of the OUD among the pregnant women and the infants by the NOWS. Across the countries the pregnant women lack to get the access to the evidence base therapies that includes the medications of the OUD, and the infants with the exposure of the Opioid that frequently received from the different cares. In the process of addiction the public systems like child welfare and the early intervention are increasing in numbers as a stretch that affected by the crisis. The approaches that are needed to make an improvement care for the mother-infant dyad. This statement provides the overview of the effect to the crisis on the mother-infant dyad and the recommendations that are provided by the management of infant with the exposure of the opioid that includes the clinical presentation and treatment, discharge and the assessment. The service that are provides by the Health

services on the Nows and primarily focuses on the treatments to the infants and with Nows. Similarly to the infants they are discharged, parents of the infants are required to give an exposure of the Opioid and the education of how to deal with the infants challenging behaviours that may increase the risks of the non-accidental trauma. (Patrick et al., 2020)

2.2. Addiction

Drug addiction is a problem and in that Opioid Addiction is the important problem in the world and it has become the important hazard among public (Salehi, Kheirabadi, Maracy, and Ranjkesh, 2011). The repeated usage of drugs in the addiction process prompts the patients to relapse into drugs followed by seeking the detoxification. The Drug addiction includes psychotropic drugs and narcotics that promotes dependency in them. These includes that the opioids can treat pain effectively but these opioids are highly addictive. If a person who is dependent suddenly stops taking those drugs, the people will show some measurable and predictable physical signs that is known as the OWS. It is found in the existing study that the traditional Herbal Chinese medicines include Oleanolic Acid that helps to reduce the Drug Addiction process and it even promotes detoxification. The Oleanolic Acid is mostly found in the medicinal herbs and food in more than 190 plants that helps about sixty families (Akkol, Goger, Kosar, and Baser, 2008). It is mainly present in form of root, rhizome saponins in the plants for the families such as the Asclepiaceae, Araliaceae, Ranunculaceae, and Curcubitaceae, when it is found in the fruits and leaves from the plants in the Amaranthaceae, Lepidaceae, Ranunculaceae, Rubiaceae and Gentiana.

In the existing study it is clearly investigated with the effects of OA in the animal models that are nal-oxone precipitated withdrawal and spontaneous withdrawal that evaluates in the effects of the Oleanolic Acid on extinction, reinstatement of the morphine that is induced in the Conditioned Place Preference, and acquisition. The Jitai tablets that are purchased from China. The Hydrochloride tablets which is used for treating the Opioid addiction that are purchased as the positive control drug from the factory in China. It is tested on 70 male mice's that is NIH which weighs 18 to 22 g, and on 160 male rats that is SD which weighs 180 to 220 g that are obtained from Guangdong medical laboratory Centre and kept under the half and half dark light cycle kept at a stable humidity and temperature also by giving sufficient food and water.

Various results are found in the model groups, the results showed that the Oleanolic Acid has some effects that is related to the alleviating symptoms of the withdrawal that improves the

levels of neurotransmitter, which also reduces weight loss. This gives the idea of Oleanolic Acid may play vital role eventually in clinical treatment for drug addiction. Though the Oleanolic Acid exhibits some therapeutic beneficial effects on the syndrome of detoxification. Existing study establishes that the Oleanolic Acid might be useful in the treatment of the morphine addiction. (Shi, Pan, Wang, and Li, 2021)

Table 1: Drugs Causes Addiction and their receptors

DRUG	RECEPTORS
Opiates (morphine, heroine etc)	Agonist at mu, delt and kappa receptors Also activate the toll like receptors
Cocaine	Indirect agonist of dopamine by inhibiting its transporter
Nicotine	Nicotinic acetylcholine (Ach) receptors
Ethanol	GABA (gamma amminobutyric acid) agonist and N-methyl D- aspartic acid (NMDA) receptor antagonist
Amphetamines	Indirect agonist of dopamine by stimulating its release
Cannabiniods	CB1 and CB2 receptors

2.3. Receptors involved in OWS

The dependence and tolerance is the main problem that limits the clinical usage of opioids for the chronic pain. There is the important overlap in the μ receptor of opioid and the dopamine pathways that have the functioning links in the brain areas. The existing study investigates if there is any adjunct therapy with the D1 dopamine receptor which prefers the antagonist or the D3 Dopamine receptor that prefers agonist pramipexole that could prevent from the tolerance of morphine and which helps to reduce the withdrawal symptoms. In the beginning the combination of the pramipexole and morphine tolerance is assessed in the mice. The mice that receives intraperitoneal morphine injections that shows the thermal thresholds that are reduced in 7 days and at the seventh day. Next the withdrawal to the combinations are tested in rats over 14 days. Those rats are assigned with one of the four drug conditions like

morphine, morphine and pramipexole, morphine and SCH 39166, and saline for the administration of chronic with the osmotic pumps. The morphine chronic administrations for the last 14 days are resulted in the important reduction of the analgesia of the morphine. The results suggested that the systemic chronic administration of either the D1 dopamine receptor which prefers the antagonist or the preferring agonist of D3 receptor as morphine to adjuvant therapy that shortens the withdrawal symptoms duration and prevents the tolerance of the morphine with the cessation of the treatment. Thus the Dopamine receptors may present the viable adjunct for the long term therapy of morphine to treat the chronic pain in clinic. (Rodgers et al., 2020)

The symptoms of the opioid which are controlled with the OWS that is significant for the Opioid addiction treatment. There are only limited evidences that is given on the pregabalin of the effectiveness on the OWS. The existing study check the effectiveness in the Pregabalin which helps to reduce the Opioid withdrawal Symptoms. The double blind clinical trial is conducted on the non-injecting users of opioid who are diagnosed with the dependence of Opioid and also referred to Addiction Treatment centre in Iran in the year of 2015-2016. The patients are divided into control groups and into the interventions, both the groups received the routine management of Bupre-norphine to the OWS. The control groups received placebos and while the intervention groups that received additionally 450 mg per day of the pregabalin. The OWS are evaluated using Short Opioid withdrawal scale, the data's are analysed in the SPss-20 and the descriptive data's are reported as the means of SD. The Analytic data's are analysed using the repeated measures of the ANOVA. The level of the statistical importance is set at the P-value.

The mean age are 41 in the group of intervention and 44 in the control groups. None of them are assessed symptoms and signs that differed importantly between the two groups. The results did not show superiority of the pregabalin of 450 mg per day that regimes versus placebo for the symptoms that controls the OWS. The further studies is administrating pregabalin higher doses are recommended. (Kheirabadi, Moazeni, Salehi, and Mahaki, 2019)

People with Opioid Use Disorder's, they need to get a longer treatment for the long action of opioid agonist like Bupre-norphine and methadone which are more beneficial usually than the taper. The Preliminary evidence that suggested the PPAR- Peroxisome Proliferator-Activated Receptors γ that are agonist the pioglitazone that reduces the symptoms of opioid withdrawal, which possibly inhibiting diseases that are increasing in the proinflammatory cytokines. Which is a randomized, placebo-controlled that are conducted in the existing's

clinical trial utilizing the two variant study designs (combination of inpatient and outpatient and entirely the outpatient) to calculate the efficacy and the safety of the pioglitazone as adjunct medication to people who are suffering from the physical dependence of Opioid they are undergoing the Buprenorphine taper. The Participants are stabilized on the Buprenorphine/ naloxone they are randomized to receive the oral pioglitazone or the placebo before, after or during the Buprenorphine taper. The measures of the outcome included in the Subjective Opiate Withdrawal Scale- SOWS and in the Clinical Opiate Withdrawal Scale used as the rescue medicine to alleviate the opioid urine positive specimens and the Opioid withdrawal Symptoms. While measuring the proinflammatory cytokines plasma and Cerebrospinal fluid (CSF) are collected in subset of participants during taper.

The trial of the clinical process was terminated prematurely because of the slow enrolment that is per group 40 participants are required to adequate the statistical power to take a test in the existing study hypotheses. Seventeen received one dose of medication in the existing study while twenty four participants enrolled. The scores of the SOWS are higher in the arm of pioglitazone than the arm of placebo after adjusting the use of the medications, the participants who are in the pioglitazone needed more rescue than the placebo during the phase of post-taper. Next the SOWS scores are correlated with the monocyte of the chemo attractant protein-1 in the CSF and with the plasma the participants who got higher level of MCP-1 reported that they are getting higher SOWS and it is mostly noted after the taper ended of Buprenorphine. From this existing study the result provided no evidence and the pioglitazone are getting reduced in the Opioid withdrawal symptoms during the taper of the Buprenorphine. The major strength of the existing study in each of the iterations was the randomized design that the placebo which is controlled. Additionally it was able to demonstrate and assess the Buprenorphine and pioglitazone's co-administration. This cannot state the categorical pioglitazone that is ineffective as the aid to taper the Opioid (Schroeder et al., 2018).

2.4. Different Stages of OWS

The opioid addiction that constitutes the importance of the contemporary health crisis which is multifarious in the complexity. The modelling epidemiology is challenging in its own right in any addiction process. The Opioid addiction challenge are exacerbated because of the difficulties that are faced during collecting the real time data and the information nature that is circumscribed about the opioid users who may disclose the owing to the stigma that are associated with the misuse of the prescription. By giving the context and identifying the

individual's progression through the Opioid addiction stages, which is one of the major acute problems in the modelling of the epidemiological solutions that are crucial to design the specific interventions in both population and personal levels. Therefore the information's are used to measure theoretic to calculate the frequency in the distribution of the generic background. In the existing model the algorithmic approach are described to address the basic challenges of detecting the various level of states that shows the usage of drugs and by recovering the data from the social media. The existing model's approach combines the neural network recurrent of learning with the analysis of information-theoretic in the word associations. The evaluations of the experiment indicated that in the approach of the existing study it can differentiate between the different stages of addiction and to identify the users who are prone to relapse with the higher accuracy as the evidence. (La Marca and Singh)

The main purpose of the existing study is to investigate the influences that happens in sex under the expression and the duration of the syndrome that is spontaneous with the withdrawal of somatic morphine. Also to characterize the relation between the cellular activation and the symptoms of the somatic withdrawal that is spontaneous in GABAergic tVTA in the female and male rats. The adult female and male long Evans rats which are Morphine-dependent underwent the spontaneous withdrawal of 72 hours and the somatic withdrawal symptoms are assessed in every 12 hours. The female rats demonstrated lower in the overall severity symptom the symptoms that are persisted in a longer time period which demonstrates the higher level of withdrawal symptom than males during the late withdrawal. The morphine-dependent male rats expressed severe symptoms while being in the early phase of the withdrawal compared to the results of the females. In the tVTA the pCREB activity are elevated in the rats that are morphine withdrawn and it is positively correlated with severity of the withdrawal symptoms. The results demonstrates the sex differences in the timing of the somatic withdrawal expression. The existing study data are added to the growing body evidence while demonstrating the role of the tVTA in the withdrawal of morphine and begins to establish the behavioural and the molecular profile of depending the sex within the brain region. (Bobzean, Kokane, Butler, and Perrotti, 2019)

In the existing study, the opioid administration is the Opioid-induced constipation- OIC is considered as one of the side effects. To address the side effect the oral peripheral of the μ receptor opioid antagonist nalde-medine is developed. Though the drug didn't affect the blood barrier of the brain which is thought that might not lead to the OWS- OWS with the symptoms of the central nervous system. The report of the cancer patient is presented with

the anxiety symptom that is centred here in the existing study and continuous irritation of four months after the nalde-medine administration for the OIC and also who are diagnosed after the close investigation with the OWS. The Sixty-five year old female patient who had surgery for the stage of the IB endometrial cancer that the patient suffered previously for four years but experienced the recurrence that involves in the pelvis two years later. The Medical narcotics are used to control the pain of the patient but the nalde-medine started to control the subsequent constipation. When OWS related to nalde-medine is suspected and the administration with the discontinued nalde-medine, the above mentioned symptoms disappeared within 2 days and there was no recurrence are found later in the existing study. (Ishida, Uchida, et al., 2022)

In this existing study, it is aimed to investigate the negative consequences of the Morphine Withdrawal Syndrome-MWS on the Conditioned Place Aversion- CPA and the response from LPGi neuron to the nal-oxone at distinguished Circadian Times-CT in the rat's that are morphine dependent. Which regards the assessment to the negative consequences of the MWS on the CPA and the Lateral Paragigantocellularis-LPGi neural activity in the rats that are dependent that are morphine dependent in the light and dark cycles. The male rats received 10mg/kg morphine or the vehicle in the consecutive days for the tests of behavioural assessment. The nal-oxone-induced PA and the physical signs of the withdrawal syndrome are calculated in the light and dark cycles. The existing study also performed in the vivo extracellular recording with the single unit for the investigation of the neural response from the LPGi to the nal-oxone in the rats that are morphine dependent on the 10th day of the morphine or the saline exposure. The results showed that the nal-oxone induced CPA in both dark and light day's cycles but the score of the CPA was higher in the light cycle. Moreover the physical design intensity of the MWS is severe in the light cycle compared to the darker cycles. The electrophysiological experiment results indicated that the nal-oxone evoked in both excitatory and the inhibitory responses in the LPGi neurons and incremental effects of the nal-oxone on the LPGi activity is stronger in the light cycle. The neurons are also with excitatory response that exhibited higher in the baseline of the activity in dark cycle, but the inhibitory response of the neurons showed higher in the baseline of the light cycle activity. The baseline firing rate of the neurons are recorded in light cycle is significantly differs in the response of the inhibitory/excitatory dependent manner. The existing study concluded that the nal-oxone induced the changes in the LPGi activity of the cellular and the behaviours of the

rats that are morphine dependent which can be affected by the circadian rhythm also the internal clock (Rahmati-Dehkordi, Ghaemi-Jandabi, Garmabi, Semnianian, and Azizi, 2021)

This existing study aims to calculate the level of the adherence to the WHO and the guidelines of the American Society of Addiction that regards the opioids addiction that medicates to identify the causes of the non-adherence in the Hamadan. The data's of the patient's are recorded with the demographics and the treatment of the process. The adherence of the mentioned guidelines are calculated and the reasons for the non-adherence are also asked from the doctors. The Methadone maintenance treatment-MMT and the Buprenorphine maintenance treatment-BMT are only common opioid addiction treatments in the Hamadan. The cases in the MMT and the BMT groups are 142 and 18 respectively. Concerning the stage of stabilization the dosage amount is altered and modified in the regular visits. In the elimination of the MMT stage the compliance is observed and there is no case for BMT which continued in the treatment of the stage. The existing study aimed to tailor the treatment by the condition of the patients or resulted from the sociocultural, financial and familiar problems. (Morasa, Haghighi, Ataei, and Rangchian, 2021)

Women with opioid misuse face drug related stigma histories that could be amplified on their pregnancy. While women were frequently blamed to their drug utilization and urged for changing, the social contexts which reinforced and created stigma were hugely unchallenged. Drawing over a multidimensional stigma model, the study (Syvertsen et al., 2021) evaluated the source of stigma manifested across the pregnancy journeys of women for shaping the access and care quality. Triangulated in-depth interviews with 28 women those with the opioid misuse history and recently gave birth (or) pregnant and 18 Ohio healthcare providers. Thematic evaluation analysed the path how the stigma operates based on the context of care. The healthcare providers are denoted as counsellors, nurses, physicians, healthcare administrators and social workers. From the 28 participants, their average age was 30, ranges between 22 to 41 years and 79 per cent of them were white. Most of the participant's utilized prenatal medication assisted treatment including methadone (or) suboxone and in that 15 were pregnant. Stigma evidence emerged across health-care contexts. Structural stigma encoded barriers towards the care in punitive drug treatment and insurance practices, where enacted stigma manifested as judgment and mistreatment from providers.

2.5. Pathophysiology of OWS

The herbal formulation compound is known as Habb-e-Shifa (HeS), which is the Unani medicinal system (Azhar, 2018) that claimed to be beneficial to the patients those addicted towards opium and support them to improve the withdrawal syndrome (Moosavyzadeh et al., 2020). The exploration has been carried out in the male Swiss Mice around the weight 20 to 30 grams. The considered animals have been grouped into five categories, each group contains six animals and induced with Morphine s.c for four days. The First group has been severed as a negative control and treated by 1ml regular Saline along with morphine. Followed by this, the 2nd group- HSA and 3rd group- HSB have been treated by extraction of HeS (50 mg per kg and 100mg per kg) for four days. The 4th and 5th group (HSCA) and (HSCB) have been treated with the same dose twice a day synchronously with morphine. All the animals, which are taken under the investigation have been received their last HeS dose extraction with Morphine, at the last day two hours before the Naloxone injection and they were estimated to number of jumps in a cylinder-shaped jar directly after the naloxone injection over a period of 30 mins. It has been evaluated that the mean ratio of jumping in HSA, HSB, and HSCA and HSCB groups were decreased significantly. The five animal groups have been compared with negative control. Testing of HeS drug resulted a significant degree of effect over the withdrawal syndrome of Morphine and hence, the validation of Unani Medicine claimed that HeS has a significant drug, which could be utilized for the opioid withdrawal management. (Mujassam, Sofi, Shabir, and Wasim, 2020)

For the constipation treatment, which is the side-effect of opioid utilization (Wild, Webster, Yamada, and Hale, 2020), Nalde-medine an oral peripheral μ -opioid receptor antagonist has been developed. The term Nalde-medine has been not typically recognized as an instigating factor for opioid withdrawal, which is correlated with the symptoms affecting the central nervous system (Yasufuku et al., 2021) . From cancer patients, those undergoing the management symptom, the author (Ishida, Hiraoka, et al., 2022) reported a case treated with Naldemdeia for the constipation treatment in association with the opioid utilization to cancer-pain and those resulted critical psychological signs correlated with sudden withdrawal action after the Nalde-medine use. A 36 years old woman analyzed with cervical-cancer level IIB, PS3. The patient utilized Oxy-codone Hydrochloride-Hydrate- 80mg per day for ileal pain has been underway on Nalde-medine for constipation, the patient endured of sweating-5mins and hallucinations after 1hr. Also, resulted some behavioral and physical abnormalities like hyperactivity, diarrhea and psychological abnormalities like aggression towards staff. In spite of the psychiatric symptoms falling over time, there were no abnormalities symptoms in

terms of blood bio-chemical data and no brain metastasis has been observed through MRI. Based on the Clinical Opiate Withdrawal Scale, these symptoms were mediated for indicating the Withdrawal of the Opioid. Nalde-medine has been discontinued owing to Nalde-medine associated OWS and then the psychiatric symptoms were reduced with no reappearance of similar symptoms detected to date.

NOWS occurred after disclosure during mothers' pregnancies with an Opioid utilization disorder (Crimmins Easterlin, Ramanathan, and De Beritto, 2022). In case of insufficient non-pharmacological treatment, pharmacological choices have been preferred, but a generally guideline for the treatment has not been determined. Sub-lingual Bupre-norphine drug has been considered as more prominent towards treatment process. Since, the oral alternatives are not obtainable in various clinics as in study unit, parenteral-morphine is still the initial choice drug. The study reported that two infants with NOWS have been efficiently treated with the combination of Buprenorphine and naloxone. Followed two infants, whose mothers had an opioid utilization disorder during their pregnancies. The modified Finnegan-Scoring Scale was utilized for the assessment of considered babies. Both infants developed persistent-seizure with resistant withdrawal signs. An effective parenteral route might not be facilitated due to the instability of hemodynamic. Hence IV morphine can't be utilized. In case of, deficiency of oral treatment alternative, initially, phenobarbital was tried up to 40mg per kg orally. After that, combined Bupre-norphine and nal-oxone tablet was utilized sub-lingually that has not been utilized before in infants. Detailed written consent has been attained from parents to the emergency utilization of these drugs in advance. Therefore, after this treatment, the withdrawal signs and seizures were controlled. There were no adverse impact and infants with complete recovery were discharged. Sublingual Buprenorphine 2 mg + Naloxone 0.5 mg in ratio of 4:1 table might be utilized effectively and without any side-effects for treating NOWS (Ulu, Kandeğer, and Meriç, 2022).

Naloxone has been utilized often and considered as an efficient treatment for reversing the life-threatening illicit opioid intoxication effect. Excessive naloxone dosing in these perspective, moreover, might lead towards naloxone-precipitated opioid withdrawal in individuals with dependence of opioid. Buprenorphine, a partial mu-opioid agonist, which increasingly utilized in the emergency department to the OWS treatment, but slight known for concerning towards their efficacy in naloxone-precipitated opioid-withdrawal cases. The author (Chhabra and Aks, 2020) reported a naloxone precipitated opioid withdrawal case, which has been efficiently treated with sub-lingual Buprenorphine. The older male with the

symptoms and signs of opioid toxicity brought into emergency department was efficiently treated with the pre-hospital naloxone through emergency medical services. The patient has COWS (or) 10 with un-intentional defecation and abdominal cramping. After a discussion on choice of the treatment and obtainable adverse effects to the patient and the decision has been made for administering 4mg per 1 mg of sublingual Buprenorphine and naloxone film. The person reported a rapid enhancement in symptoms and post-treatment was done after 30 mins, the COWS was four. COW's rate was decreased after 1 hour to 3 and this was sustained for other 4hr observations. The patient has been consequently discharged to the ability of the treatment to opioid utilization disorder. This particular case highlighted the Buprenorphine potential a treatment of modality to acute naloxone precipitated withdrawal of the opioid.

2.6. Role of Toll Like Receptors (TLR) in OWS

The toll like receptors are considered to be the significant mediators of the inflammatory pathways in gut which plays a major role in intervening the immune responses towards a huge range of pathogen driven ligands and the relation of adaptive immunity with the innate immunity. The particular part of the brain could cause addiction namely the mesolimbic dopamine pathway. It is also be referred as the reward circuit of the brain. Studies (Wu and Li, 2020) have reported on the important roles of TLR gene among different populations. It holds the position of providing first line of protection against the pathogens because of the ability to recognize the pathogen linked molecular patterns. It also could have conserved the structures and damages that has been caused by the pathogens within the host. The study has emphasized the focus on the neuronal adaptations and alterations of TLR due to drug addiction. It has considered research on the role of innate immune systems particularly TLR4 signalling particularly in drug addiction associated behaviours.

Specifically, drugs like opioids activate TLR4 signalling and consequently induces pro inflammatory responses which again contributed in the development of drug addiction. The inhibition of TLR4 could strengthen the effect of opioids but this also could involve in the withdrawal behaviours of various drug classes. The results from the study has shown that TLR4 in relative to immune responses has played only minor part in the role of drug addiction. It has taken TLR4 signalling involved in various drug classes and maximum actions that underlies with this effect. It has been reported that opioids like morphine could be able to induce neuro inflammations in the central nervous system.

This kind of neuro inflammation has been always correlated with the effects of drug dependence, analgesia, tolerance and withdrawal. The study has found that drug morphine could be binded directly to the differentiation of protein myeloid. The isomers consisted with naloxone in the constitution of 26.3 mg has been found to be inactive at opioid receptors. The results from the analysis has shown that through TLR4 activation with the satisfying effects of opioids. Additionally, the microdialysis from the study (Thomas et al., 2022) has shown naloxone has reduced morphine prompted boost of dopamine amount in the case part of NAc (nucleus accumbens). Altogether, it suggests that TLR4 with MD-2 of signalling combined with the opioid receptors could mediate with the opioid reward behaviours.

However, the study (Bruno et al., 2018) has contradicted on the TLR4 in opioid addiction and has reported that either naloxone or naltrexone could inhibit the induced TLR4 activation into the vitro. The morphine could inhibit the activation in TLR4 in the manner of concentration reliant and hence the consequence cannot be changed by naltrexone. The non-existence of both naltrexone and naloxone in research have considered the absence of biotransformation within the systems. Different form various methodologies, it has mentioned that nor every agonist and antagonist relationships seems to be equal under various conditions.

The antagonist will not bind to receptors if it finds to be completely occupied by the respective agonists. There could be various signalling pathways that could have determined the exact signalling which has been underlying on the test agents. The mixed outcomes from the vivo research requires still more consideration on the TLR 4 mutant and opioid induced tolerant, physical dependence and hyperalgesia. The study on (Liu, Li, and Wu, 2022) has suggested on the role of TLR4 in opioid activity. The chronic dose of naltrexone instantly before the extinction assessment procedure had not any seeking outcome in opioid craving behaviours. The severe provision of naltrexone could not cause heroin access behaviour. It has been found that the naloxone or naltrexone could also reduce the food self-management which has suggested the absence of TLR4 behavioural specificity in antagonists on maintaining drug related operant behaviours. It has provide the TLR4 (+) selectivity of isomer-ligands and have recognised on the non-stereo selective activities in naloxone on positions excluding TLR4. In order to predict the particular process carried by TLR4 specifically in the addiction of opioid, the study has examined on the isomers acting on TLR4.

The studies (Zare, Pourhadi, Vaseghi, and Javanmard, 2022) has indicated that TLR in brain specifically on microglia responds to the essential immune agonists like micro RNAs along with HMGB1. Numerous TLRs are induced into the brain by the form of alcohol consumption, drug abuse or by stress and increased to the post-mortem of human brain. The improved TLR innate immune response signalling in brain could lead to the loss of neuronal cell populations, epigenetic modifications or alterations in the synaptic plasticity. It together contributed in the emotive and cognitive malfunctions. The addiction generally involves with the increasing stages of drug overdoses and in toxification, negative effects of withdrawal and finally compulsion in drug usage and drug abuse. TLR signalling within the CLC (cortical-limbic-circuits) are changed by the drug and depression in the way which is reliable with the stimulating progression done by various phases of addiction.

The drug abusing activity could contribute in progressive initiation of essential immune pro inflammatory responses. The stress inducing innate immune responses was found to be useful or maladaptive outcomes on the functions in brain. The stressors like infection and also injury could tempt the cytokines with other signalling-molecules which could activate behavioural variations referred to the illness behaviour. Such kind of behaviours are included with the social withdrawal, sleepiness, decreased interest in activity which require adequate facilitations of energy conservations and recovery from illness. It also has been found that the psychological stressors can also induce inflammatory responses. The response over the sickness behaviour which is adaptive in nature also leads to acute inflammation or stress and prolonged exposure to inflammation could also leads to maladaptive which in turn directs to the neuro-psychiatric disease called as depression. The similarities in activity of sickness behaviour and depression seems to be same. Chronic activation of innate immune followed by the drug abuse could result in the psychopathologies. Hence, the consistent innate immune inhibition caused by chronic drug could also direct to the emotive ad cognitive malfunctions.

The peripheral inflammatory responses (Araldi, Bogen, Green, and Levine, 2019) could impact the brain and its behaviour over various routes. The modifications in cytokines systematically with the receptors placed on the vagus nerves. The vagal activation would be communicated to neural-centres inside the brain for promoting the stimulation of illness behaviours. The sickness behavioural responses into the injection of TLR4 agonist is performed through vagotomy. The cytokines have influence over the brain through the transportation in blood. The pro inflammatory cytokines produced within blood could be diffused through the penetrable sections of blood and brain-barrier. Transporters of TNF- α on

blood-brain barrier are important for systemic inflammation to cause the brain inflammation. In the same way, in humans TNF- α circulating could alter the availability of the brain serotonin transporter and the activation of such immune signalling could cause on the vaccination such as fatigue, confusion and loss of motivation. The fatigue in humans induce the alterations in the microstructure and cortical-metabolism of cortex. The triggered immune-cells like monocytes could be transferred into brain. After activation into the brain, essential immune-response continues which contributes to the addiction of drug consumption. The consequential discharge of inflammatory-cytokines particularly from part of liver could activate the essential immune structure in brain with the straight transportation with the cytokine receptor and stimulation of vagus-nerve. Drug acquaintance leads to essential immune stimulation into the brain which induces the brain malfunctions of memory loss, fear learning and also advances in the stages of addiction.

2.7. Role of Tumor Necrosis Factor alpha (TNF) in OWS

Tumor Necrosis Factor alpha (TNF) is the inflammatory cytokine that produces more than one effect on different cell kinds through wide range of signalling events among the cells. It leads to the programmed form of cell death and has been recognised as the primary controller of inflammatory responses which is involved in the pathogenesis of autoimmune diseases. It is considered to be the main component of opioid tolerance. TNF-alpha plays significant role in the critical stage during development of abscesses in brain through regulation of anti-bacterial inflammatory responses. The research (Eidson and Murphy, 2019) has taken the study on the structure and function of TNF-alpha and discussed insights on its pleiotropic effects on both malignant and normal cells. Protein is significant since it could act as the agent of resistance to cancers and infection. The study has shown that TNF alpha has exerted many effects through binding cell membranes and as the trimmer to both 55 KD a membrane receptor referred as TNFR-1 and 75 KD a -receptors named as TNFR-2. Both the receptors fall under the TNF receptor family. The family structure of receptors act as the communication line that helps in the activation of pathways due to cell death or assist in inducing the gene expression involved in survival or cell differentiation. The study has analysed the numerous relative decoy receptors which has helped in the isolation of TNF molecules that resulted in the rescue process of cells from the necrosis or apoptosis. It has defined the crystal structures of TNF alpha, TNF beta and added cellular structure of sTNFR-1 through crystallography.

The recommended study (Parekh, Paniccia, Adams, and Lysle, 2021) has identified numerous people with multiple chronic conditions of OUD and post – traumatic stress disorder. Since TNF- α is considered to be the integral component of learning and memory processes in brain, the study has researched on TNF alpha signalling that mediates certain long lasting maladaptive behavioural responses induced by the chronic heroin and withdrawal. It also has shown (Zazula et al., 2022) that excessive exposure to chronic heroin administration and withdrawal will induce immune reactivity of TNF alpha and disrupted the DH TNF- α signalling during the stage of withdrawal that blocks the enlargement of enhanced fear learning. The research has identified that fear learning occurs through drug abuse and its withdrawal which has been determined by the hippocampal-TNF- α signalling since through inhibition of DH TNF- α signalling that interrupts the further exaggeration of fear conditioning. Through this study investigation, it has been predicted that through intra-DH fusion in TNF-alpha inhibitors approximately 48 h into the drug withdrawal has prohibited the progress of fear learning and has also minimized the weight loss due to opioid withdrawal.

Patients suffering with the OUD will have impacts of memory deficiencies and outcomes due to improper treatment. The studies have evident that abusing the drug opioid will activate inflammatory actions by increasing the production of cytokine and impairment of neuro protection could damage the function of memory in patients with OUD. So, the study (Wang et al., 2018) has predicted whether the plasma based inflammatory and neuro trophic markers have correlation with the memory actions in OUD suffering patients. The recommended research (A. B. Srivastava, Mariani, and Levin, 2020) has undergone MMT (Methadone Maintenance Therapy) which has been investigated and followed up through 12 weeks. It has found that changes in the level of inflammation has been involved with the memory activities of patients with OUD. The study has hypothesized that immune process in brain involved with cytokine production or microglial activation have pivotal role in memory, learning, excitability and neural plasticity. Proper control and timely activation over the immunes could mitigate the disturbances in both memory and neural plasticity. But in contrast, if the immune processes were severely activated, then impairments in learning and memory has increased. Hence the study has shown that greater levels of inflammation have impacted negatively on memory test scores. The relationship has found negative correlation between the TNF- α level and memory indexes by controlling through multiple factors. The findings

from the study has indicated that TNF- α is the pathway involved in the memory processes of OUD patients.

The study (Pergolizzi Jr, Rosenblatt, Mariano, and Le Quang, 2019) has taken evidence on the fact that severe opioid exposure has induced glial activation and pro inflammatory mediator expressions in Periaqueductal gray (PAG) brain region which is closely associated with the complex syndrome of dependence and withdrawal of opioid. Particularly, through increasing of TNF- α on glial cells which has been activated through opioids that directly impacts over the neural operation of PAG followed by the alteration in the gene expression and symptoms of precipitation of withdrawal after the discontinuation of opioid. The glial cells are closely involved in the active control of neuronal activity. The inhibition of biological function of TNF- α has suppressed the removal of chronic morphine and has reversed the neurochemical responses. The study has revealed that interactions among glial neuronal mediated through the interactions of PAG glial TNF- α with TNFR has played significant role in the opioid withdrawal syndrome. Thus the findings from the research has resulted that the inhibition of TNF- α would represent a new approach in the path to preventing opioid withdrawal. The study also has demonstrated that withdrawal from morphine induces astrocytic activation in order to release TNF- α in PAG. Through injection of TNF- α arouses morphine withdrawal like behaviours. Thus TNF- α could play major role in the glial neuronal interactions which influences drug abuse. Opioid dependence leads to severe negative emotions which are associated with the withdrawal syndrome. Such states of negative emotions generally emerge from the activity in amygdala which is the part of the brain involved in the emotional and behavioural responses. From the suggested study (O'Sullivan et al., 2019), it has been inferred that the nucleus of amygdala has implicated strongly by the opioid dependence. It also has measured gene expression in single glia and neurons being gathered from the amygdala with the laser capture dissection method and subsequently has measured gut microflora in the phase of morphine dependent and withdrawal in order to predict the factors of negative emotions in opioid withdrawal.

It also has found that up regulation of TNF in the withdrawal state has been performed and observed that the ratio of Firmicutes and Bacteroids in opioid withdrawal has indicated gut dysbiosis. It has been inferred that changes in gut microflora and inflammation has contributed to the negative emotions which has been driven by the opioid withdrawal. The study has confirmed increased level of TNF- α protein with the methods of western blot and immunofluorescence. The results from the analysis has shown that each kind of cell had

increase in the TNF expression in withdrawal through which microglia has shown increase from placebo to morphine. The study has confirmed that TNF- α protein was available at higher levels in withdrawal and lower levels in morphine. TNF- α protein signal was presented in the amygdala region.

The study (Alvarez Cooper, Beecher, Chehrehasa, Belmer, and Bartlett, 2020) has examined on the cognition immune interactions such as functioning memory, executive functions, TNF- α and level of receptors in BD (Bipolar Disorder) affected patients compared with controls. The neurocognitive examination has been performed for the task of working memory and executive functions. Symptoms of chronic depression and mania has been assessed through the study. The results have shown that the performance on working memory was found to be worse with the higher levels of TNF- α compared to controls. TNF- α along with its receptors was found to be the significant variable in the impairment in BD. The outcome from the study has presented correlation between the neurocognitive tasks which has evaluated working memory, serum levels of TNFR 2 and executive functions within the individuals suffering with BD. It also had found association between the TNFR1 with the symptoms of anxiety, depression and manic state. Thus it has been hypothesized that anti TNF- α agents was considered as potential adjunctive therapy for BD patients with the high level of TNF- α and its corresponding receptors that prevents neuroprogression.

2.8. Drugs used in the treatment of opioid withdrawal (synthetic drugs)

Opioid use disorder is a kind of chronic disease similar to other forms of diseases like asthma, diabetes and hypertension. Through medication form of treatment (Edwards et al., 2020) considerably decreases the probability of overdose that allows many people to bounce back to their normal life. The treatment for opioid is involved with antagonist

- Naltrexone,
- Methadone and
- Buprenorphine.

Every medication has both effects of advantages and disadvantages. Naltrexone is the drug requiring complete detoxification and has high rate of discontinuation. Since it reduces tolerance, it also enhances the risk of mortal overdose in circumstances of relapsing frequency. Methadone drug is considered to be best in preventing relapse but also has the side effects of overdose. The patients have to visit the clinic regularly during intake of this kind of

drug. The partial agonist buprenorphine usually be prescribed by the medical advisors and can be consumed while sitting at the office or at home. Death due to overdose of buprenorphine are relatively less compared with heroin.

The study (Clemans-Cope, Winiski, Epstein, and Basurto, 2020) has assisted the performance of drug with patients under medical supervision. It has taken 80 patients who were considered to be eligible for the research. Thirteen of them refused to follow the information and still five more were escaped before evaluation and with the total of 62 persons, the study was conducted. Out of 62, only 53 have met the criteria for inducing buprenorphine and nine person has not met criteria due to lack of withdrawal symptoms or due to very recent addiction of opioids.

Even though drugs for opioid withdrawal was appeared not to be life threatening, the clinical analysis can make chronic fluid loss or some kind of electrolyte abnormalities which could result in the haemodynamic instability and even to death. The withdrawal process from certain addiction substance is generally characterized in the statistical and diagnostic path for mental disorders. The OWS is the collection of clinical symptoms included with the mydriasis, piloerection, tachycardia and hypertension, insomnia, nausea, vomiting.

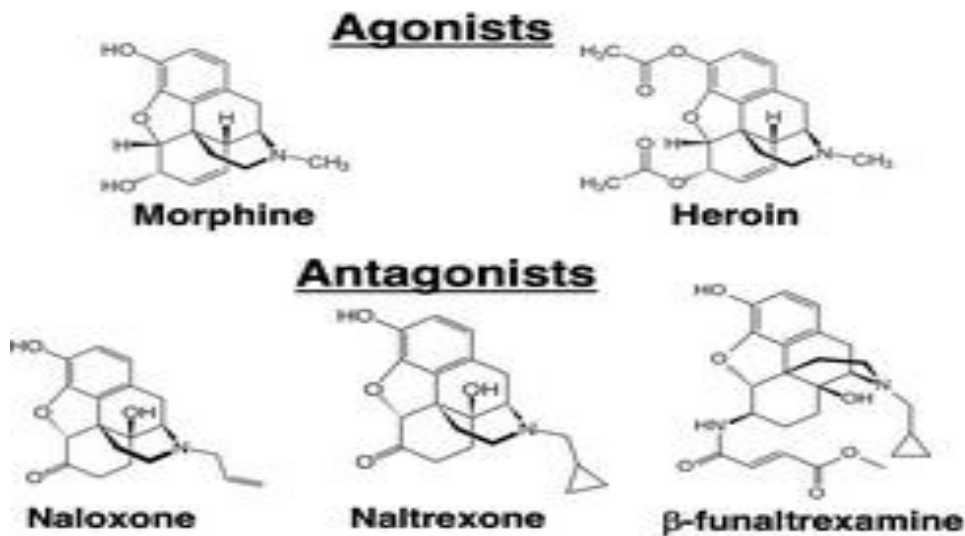


Figure 1. Agonist and antagonist drugs

The medications that are commonly used for treating opioid abuses the brain cells similar to the way of addictive opioids. Methadone could stimulate the cell similar to other opioids but has different effect due to the different action durations. The agonist form of drug usually binds closer to the receptors which produces responses in the similar way as the planned receptor and chemical. The antagonist drugs also binds to the receptors on its primary site or on other sites which all combined together that stops the receptor from generating responses. (Volkow, Jones, Einstein, and Wargo, 2019) The FDA (Food and Drug Administration) has approved the above-mentioned drugs for medication assisted treatment for OUD. Methadone has been considered as full agonist in the study and buprenorphine was considered as the partial agonist and naltrexone was the antagonist. Medicines for OUD were generally given based on the behavioural treatment. Medicines for OUD enhances the social functioning while at the same time minimizes the risk of infectious disease transmission, overdose and retaining in treatment for a longer period of time. Treatment for agonist and antagonist were different from the sustainment of addiction to opioids or other forms of addiction. The slow level entry of buprenorphine and methadone into the brain reaches certain degree of satisfying effects. It provides relief from the withdrawal and craving and also does not produce intense joy when has been injected with other opioids or at high level of doses. The slow approach of medicines provide stability and provides re-entry into their normal life with the integration of their communities.

2.8.1. Buprenorphine

Buprenorphine (Toce, Chai, Burns, and Boyer, 2018) is the drug which is utilized for the dealing of opioid dependence. It is commonly formulated with the naloxone which act as the deterrent to intravenous abuse of medicines that induces opioid withdrawal quickly. The antagonism of the receptor paves way to minimized dysphoria and miosis through inhibiting hormone release. Oral supervision with naloxone will not induce in the withdrawal of opioid. It is the partial μ - opioid receptor with high binding affinity. It has been considered to be the partial agonist of mu receptor which has partially activated opiate receptors. It is strong at mu but has been weak at kappa and delta receptor agonist. The powerful analgesic acts towards the central nervous system. The unique quality of buprenorphine is its partial agonism. The unique properties are particularly at the analgesic effects on the higher doses and effects after antagonistic. It has its maximum effects on the respiratory depression which is meant to be safe than methadone in treatment of agonist substitution in addiction. It has higher affinity binding of mu-opioid receptors and slower detachment kinetics. Through the similar way, it

has been differed from the fully opioid agonists such as fentanyl and morphine which could allow withdrawal symptoms which is milder and less comfortable for the patient.

When medication has been taken orally, it has reduced bioavailability due to its initial passing effect. The intestine along with liver break down plays majority of the drug. The absorption will be completely fast and could avoid the first pass effect. The slower action begins after placing the medicine under tongue and rises to its maximum effect after 3 to 4 hours of administration. Usually buprenorphine will be broken by the enzyme named cytochrome CYP 3A4 into an active metabolite with the weak intrinsic actions. Buprenorphine has the average half-life of about 38 hours which has been followed by the sublingual administration. The majority of the drug could be excreted through kidneys which excretes less than 20 %. Due to the capability of its slower and prolonged duration of reactions, the buprenorphine drug is found to be helpful in the treatment of opioid dependence. The medications will be provided on alternate days unless the patient gets stabilized over the daily utilization.

2.8.2. Methadone

Methadone (Mojtabai, Mauro, Wall, Barry, and Olfson, 2019) is the receptor agonist used mainly in the management of OUD. It was found to be efficient in holding of people in the medical treatment of plans that decreases the illegal use of Opioid and minimizes the causes and mortality rate due to overdose. It was used as the treatment for the abstinence-syndrome. The measure of methadone is approximately 15-30 mg per day required for controlling and minimizing the side effects and withdrawal symptoms. It is traditionally prescribed from clinics through which patients must intake medications every day as per the prescribed doses. In spite of the benefits being proved in treatment of opioid use disorder, the lack of support from community, the sparsity in spaces and the social-humiliation has continued to bound its usage. It is provided as the combination of two-enantiomers with the R-methadone that possess the affinity for μ - opioid receptor.

It is considered as the complete opioid agonist. The methadone formulations (Ledgerwood, Lister, LaLiberte, Lundahl, and Greenwald, 2019) reach the maximal concentration which is highly protein bounded limiting to the elimination of extra corporeal as the treatment of overdoses. The respiratory illness or depression occurs due to the overdose usage or illicit utilization at extreme agonism of (μ - opioid-receptors). While buprenorphine has maximum

effects that limits to euphoria or respiratory illness, no such kinds of ceiling effects occurs to methadone. The patients who initiates opioid-substitution treatment are at high risk of death specifically during first four weeks for treatment. The hazard occurrence of death also gets improved after discontinuation of treatment.

2.8.3. Naltrexone

Naltrexone (Oesterle, Thusius, Rummans, and Gold, 2019) is the medication assisted treatment for the opioid use disorder. Naltrexone is considered to be the opioid antagonist which could block sedative and euphoric effects of opioids like morphine or heroin. It also could reduce the desire for opioids. It has the capability in the reduction of dependence factor on opioids through various actions in brain in such a way that it could block the brain's receptors which has considerably reduces the longing and urge to intake the opioid. There were certain things during utilization of naltrexone for the opioid use disorder. The person (Saucier, Wolfe, and Dasgupta, 2018) who has used must have to withdraw from the opioid before 10 days in starting the naltrexone since due to severe symptoms occurs during opioid withdrawal. Abruptly stopping the intake of naltrexone could lead to serious damages and the timeline provided by the health advisor has to be followed. The person under the medication of naltrexone should be under the supervised medical care who could ease the discomfort. During the treatment of naltrexone, the person has to inform the medical professional about the medicines that they have taken for some other treatment of diseases. During the intake of naltrexone, the person should stay away from the usage of alcohol, tranquilizers, sedatives and other forms of illicit drugs. Naltrexone treatment has been involved with the behavioural counselling and therapy (Benth et al., 2019).

2.9. Drugs used in the treatment of opioid withdrawal (Herbal Drugs)

2.9.1. Ibogaine

Ibogaine is an alkaloid with strong effect in opioid withdrawal. It has significant affinities for multiple binding sites within central nervous system, including NMDA, kappa opioid and nicotinic receptors. It reduces the withdrawal symptoms of opioids like irritability, sweating and chills. Craving is an important symptom contributing to continued drug use by addicts. Opiate-dependent subjects report increased drug craving during the early stages of withdrawal (Best *et al.*, 1996). It was reported that drug craving for opiates is significantly decreased in patients of opioid withdrawal when they were under the treatment of ibogaine.

Large dose of ibogaine in the treatment of opioid withdrawal causes ataxia followed by xerosomnia (Greshon and Lang, 1962).

2.9.2. *Melissa officinalis*

Melissa officinalis, (Zargari A, 1989) has analgesic, antianxiety, sedative, antispasmodic, and hypnotic properties (Heidari et al., 1998). Aqueous extract of *Melissa officinalis* branches on symptoms resulting from morphine withdrawal in male rats, the extract of *Melissa officinalis* with dosages of 10 and 25 mg/kg, dose dependently had significant reduction on the number of jumps and defecate weight, 30 minutes before naloxone injection, in comparison with control group (Miladi et al., 2008)

Melissa officinalis, through reduction of the activity of serotonin and binding to γ -aminobutyric acid A (GABA A) receptor, reduces anxiety and in this way offsets the signs of morphine withdrawal syndrome. It has been shown in a study that intracerebral and intraperitoneal injection of GABA A, agonist receptor decreased the naloxone-induced jumping in morphine withdrawal syndrome in mice (Yoon et al., 2007). It is likely that some of the observed effects of *Melissa officinalis* extract are induced by binding to the GABA A receptor.

2.9.3. *Ferula persica* L

Ferula persica L (Miladi et al., 20210) has different effects on nervous system, including analgesic, anticramp, and antispasmodic activities on ileum contraction (Yoon et al., 2007). Hydroalcoholic extract of aerial parts of *Ferula persica* L in dosages of 50, 100, and 200 mg per kilogram of body weight did not decrease the number of jumps resulted from naloxone injection in addicted rats compared with the control group. However, antispasmodic action of the plant on ileum causes decrease in defecation in animals (Mandegary et al., 2004)

2.9.4. *Avena sativa*

Avena sativa is a sedative and neurotonic and is used in traditional medicine to treat anxiety and insomnia, especially in addicted subjects (Nasri and Shirzad 2013). The combination of the alcoholic extract of *Avena sativa*, *Hypericum perforatum*, *Passiflora incarnata*, and *Lavandula officinalis* (post and cotreated) have been shown to significantly reduce morphine withdrawal symptoms. Administration of the extract cocktail prior to naloxone

induced precipitation of withdrawal syndromes also reduced the expression of syndrome signs (Karimi et al., 2008).

2.9.5. *Lavandula officinalis*

Lavandula officinalis has antispasmodic, antidepressant, and sedative effects and reduces withdrawal symptoms (Zargari 1989 and Jadidi et al., 2011)

2.9.6. *Hypericum perforatum*

Hypericum perforatum, other than being an anti-addictive, is a sedative and neurotonic plant (Akhondzadeh et al., 2001). This plant is used to treat neurological diseases, especially depression (Barnes et al., 2001). In a study performed in 2009, inhibitive effect of this plant on morphine withdrawal syndrome was investigated. According to these results, behaviors of morphine withdrawal syndrome, including jumping, standing, and bruxism in rats were significantly decreased when *Hypericum perforatum* extract was used along with morphine consumption (Adams et al., 1995) Spasmodic, analgesic, and antimigraine effects.

Hypericin in the plant inhibits the enzyme activity of monoamine oxidase and is used to treat depression. The most authoritative scientific works such as basic and clinical books, including Martindale and PDR (Physician's Drug Reference), approved the antidepressant effect of this plant. Hypericin in this plant inhibits the activity of monoamine oxidase, inducing antidepressant activity.

2.9.7. *Withania somnifera*

Withania somnifera is commonly used in Eastern countries, Africa, and India to treat diseases, including neurological diseases (Kulkarni et al., 2008). According to a study in 2009 the use of this plant with morphine in male rats decreased symptoms resulting from morphine withdrawal (Kasture et al., 2009).

2.10. Drug Review on Pirfenidone

Pirfenidone suppresses the pro inflammatory cytokine TNF- α through the translational mechanism which was independent of the activation of the mitogen activated protein kinase and N-terminal kinase. Pirfenidone (Walker and Margolin, 2001) is the small chemical molecule which is very stable and could be easily soluble in the form of lipid-solvents and also could be soluble in the distilled water. The concentration of pirfenidone from gastro

intestinal-tract after the oral absorption was found to be efficient in animals. The drug will be quickly metabolize into the liver and could be excreted along the metabolites in urine. For ordinary humans, even after oral consumption of single dosage, effective serum levels of this drug will be seen within 30 minutes. The study (Zhang et al., 2021) has evaluated whether the pirfenidone drug can be administered through the oral course which could be arresting the evolution of most common irreversible-neurological-disabilities which are closely connected to the secondary advanced multiple sclerosis. The study has performed repetitive assessment on the physical disability and incidence of relapses and has been observed through the brain magnetic resonance imaging. The study has performed the treatment of secondary progressive multiple sclerosis which is insufficient in stabilizing the incapacities that are related with the disease.

The pirfenidone drug is the non-peptide drug mainly utilized for the decreasing the synthesis of TNF- α and also the block-receptors for TNF- α . Since TNF- α is assumed to be the cytokine in the process of demyelination, the research on the oral form of pirfenidone was done to patients over a period of 2 years. The research has taken patients under medical supervision to ensure the effectiveness of the reactions and complications through intake of drug. Certain form of stabilization and improvement (Oku, Nakazato, Horikawa, Tsuruta, and Suzuki, 2002) has occurred among patients and evaluated the primary and secondary measures. The drug has offered defence and safety over the slow level advancement of the disease. Many patients have practised the cumulative reduction in the neurological-disability. Findings from the study has indicated that pirfenidone could inhibit the formation along with the pharmacologic activities of cytokines. Particularly, it inhibits the process of synthesis and also the release of TNF- α from various cells of human-astrocytes. It also has inhibited or blocked the toxic effects on normal cells applied by the numerous tissue-levels of TNF- α .

TNF- α is considered to the significant component of the inflammatory responses which is linked to the various states of diseases. Certain new treatment strategies help in the reduction of circulating TNF- α either with the neutralization of anti TNF- α binding proteins through drugs that could inhibit de novo synthesis of TNF- α like pirfenidone. The recommended study has examined on the effect of both varieties of drugs on cell related or secreted TNF- α produced by THP-1 cells. All the drugs has significantly reduced the secreted amount of bio active TNF- α following the stimulation which was measured through bioassay. But the etanercept treated cells had six fold greater levels of cell linked TNF- α . Pirfenidone has reduced both the cell associated and secreted levels of TNF- α .

2.11. Drug Review on Piperine

The usage of narcotics drugs like opioids and non-narcotics drugs such as corticosteroids and salicylates for pain and inflammation management have various side effects on human health. Hence the search for natural solutions emphasised the identification of Piperine, a plant based drug showing excellent functions against cyclooxygenase-1 and 5-lipoxygenase. Piperine drug has the ability to inhibit the TNF-alpha induced reactions in human brain. Piperine also has the capability to modify drug metabolizing enzymes, disorders due to gastrointestinal tracts and drugs bioavailability. Piperine is a class of amide alkaloid which provides multiple phenotypic attributes such as anti-inflammatory, anticancer, antioxidant, neuroprotective, hepatoprotective, antihypertensive and enhancer of fertility and bioavailability based functions. Piperine is the major component extracted from *Piper nigrum* (black pepper). A climber and a flowering vine species of plant produces the spice black pepper. Pepper is the native species of South East Asia and comes under the family of Piperaceae. Peppercorn constitutes multiple medicinal values and is said to be “King of spices” and “Black gold”.

Pepper has the ability to function as healer of laxative, stomachic, carminative and anthelmintic diseases, enhances appetite, helpful in bronchitis, spleen diseases, tumor, abdominal pains and ascites. It also acts as a relieving agent in various diseases associated with respiratory tracts like asthma, cough and bronchitis as an analgesic agent. It also can be externally applied for inflammation and muscular pains. Pepper has various medicinal values such as douse in drowsiness and coma, in bile duct and gall bladder issues as cholagogue, as antihelmetic agent in leprosy, etc. The natural agent of Piperine is green, white and black pepper. The other major components available in pepper are piperlylin A, pipericine, chavicine, piperettine, piperanine, piperolein B and piperettine. Piperine belongs to compounds of vanilloid class.

Piperine is a nitrogen containing alkaloid agent isolated initially from pepper dried fruit extract in the yellow solid crystalline form in 1820 by Hans Christian Orstedt, a Danish Chemist. Piperine was extracted by shade dried and steamed black pepper, grounded to eliminate the volatile oils. From the grounded pepper, oleoresin, a solvent portion is extracted repeatedly by class of organic solvents like acetone, ethyl acetate, ethanol or dichloroethane. Oleoresin constitutes flavour, pungency, odour and contains about 13% of black pepper. Subsequently, the solvent is removed by subjected to pressure and recrystallized to extract crystals of pure piperine. Chemical extraction of Piperine was done by the reaction of piperidine with piperic acid chloride in 1894 by Scholtz and Ladenburg. The molecules of

piperine contains conjugated chemical aliphatic bonds, that function as interlink among 5-(3, 4-methylenedioxyphenyl) and piperidine. Piperine has received significant attention during the previous decade for its great biological use.

The structural alteration of Piperine can provide different bio-active functionalities in the domain of drug discovery and medicinal chemistry. The study (Sivashanmugam and Velmathi, 2021) has been done to analyse the significance and role of natural medicinal products using standard molecule like morphine and structural alteration of Piperine. The anti-microbial and GABA modulation of Piperine drug has been analysed elaborately in the study to determine its feasible molecular pharmacophore. Piperine, an active alkaloid component found in black pepper constitute various anti-microbial effects and can be used as pro-drug due to its Lipinski's rule. From the analysis, the piperine molecule having MDP ring and Catechol, an alkene chain and C=O bond have been proposed. Even though various analogs of both applications were found, there still exists a lack for proper framework. The piperine tested for anti-cancer drug could prove significant results with proper implementation. Various clinical and preclinical studies have been performed on the biological functions of Piperine.

A research (Dhargawe, Mahakalkar, Mohod, and Raj, 2021) has been conducted to analyse the antipyretic, analgesic and anti-inflammatory functions of piperine in analogous to aspirin. The male and female mice of Swiss family with weights around 25-30 gram and Albino Wistar rats having weight around 200 gram has been used for investigation. To evaluate the analgesic function, the hot plate and tail-flick model has been used in rats and writhing model induced by acetic acid has been used on mice. The anti-inflammatory functions have been analysed by the application of arthritis methods induced by formalin, granuloma method induced by cotton pellet and paw edema model induced by carrageenan methods. The evaluation of antipyretic function was done by Pyrexia method induced by Baker's yeast. The results of evaluation showed significant analgesic control function through oral drug administration of piperine. Although the result analysis pattern provided higher rate of function with aspirin. The anti-inflammatory analysis results proved the statistical significance with acute inflammation by paw edema model of carrageenan and chronic inflammation by arthritis induced by formalin method. However, the granuloma method induced by cotton pellet failed to contribute towards anti-inflammatory function of sub-acute inflammation. Hence further studies to administer the oral dosage of Piperine in anti-inflammatory and anti-pyretic agents can impact the significance of the drug. Even though,

piperine has proved to be a potential anti-inflammatory, analgesic and antipyretic agent, the evaluations results exhibits higher functionalities with aspirin. Further the antinociceptive and anti-inflammatory functions of Piperine has been analysed with the below study.

The piperic acid obtained from the molecule of Piperine, an active component of pepper was evaluated(de Almeida et al., 2018) to analyse its anti-inflammatory and antinociceptive activity of the compound. The anti-inflammatory effects of PAC was analysed using air pouch tests, in vitro COX inhibition assay and paw odema tests. The antinociceptive functions were analysed through tail flick test, abdominal writhing, capsaicin and formalin tests. The generation of IL1 β and TNF- α and Cholinesterase assay have been analysed to evaluate the mechanism of PAC by tail flick test using atropine, glibenclamide, naloxone and L-NAME. Also it proved that nociceptive effect has been reduced significantly by induction of formalin or acetic acid. PAC reduced the antinociceptive effect by atropine, antagonist and muscarinic receptor using tail-flick model. TRPV1 involvement in capsaicin-induced nociception demonstrated the inhibition by PAC. The molecule did not interfere in the nociceptive response of animals and hence did not alter the animal's motor capacity. The results of the study provided that vanilloid agonist and cholinomimetic action of PAC were responsible for anti- inflammatory effects and antinociceptive devoid of subchronic and acute toxicity. The results of toxicity model of Piperic acid without behavioural changes demonstrated 30 times higher dose used than VIVO experimental models. Also the alteration of organs were not found which indicates safe usage of PAC. To obtain a clear view of molecular mechanisms, more studies have to be conducted.

The piperine activities in phytochemicals and drugs enhancement, brain penetration functions in prevention, cure and management of diseases and disorders can be analysed (Tripathi, Ray, and Mishra, 2022) with view of clinical and preclinical data, extraction mechanisms, nano formulations, molecular docking and structure activity relationships. Piperine has provided significant results on preclinical actions against numerous human diseases like inflammatory and cancer treatments and also in in diabetes, oral cancer, arthritis, obesity, multiple myeloma, breast cancer, Parkinson's disease, cardiovascular disease and Alzheimer's disease. Various molecular targets for DAB-2 gene in TGF- β pathway for chronic kidney diseases remain unexplored. The pleiotropic functions of piperine related to its functional abilities in controlling various signalling compounds such as anti apoptotic proteins, cell cycle proteins, cytochrome P450 3A4 and even corona virus. Computational results has been done on nano formulation with piperine for SH-SY5Y cells metabolism activities in human. The results

have been found satisfactory to provide enhanced antioxidant effect on Alzheimer's disease. However, the research does not suggest Piperine for human use as the study exhibited that Piperine shows significant changes in human metabolism activities.

3. HYPOTHESIS, AIM AND OBJECTIVES

3.1. Hypothesis

3.1.1. Hypothesis for choosing the Tumor necrosis factor- α for the current research

From the review of literature, it was observed that after the chronic use of opioid drug, morphine, it increased the level of TNF- α by the activation of toll like receptors specifically TLR-4 and causes the opioid withdrawal syndrome like stomach cramps, anxiety, depression, craving for the drug etc.

Neurological areas that are involved in the treatment of addiction include periaqueductal gray (PAG), the locus coeruleus (LC), amygdala, ventral tegmental area (VTA), nucleus accumbens (NAcc) (McPhie and Barr *et al.*, 2009). Severe introduction of narcotics stimulates the glial cell in the spinal cord, hippocampus and PAG. Opioid persuades inflammation in the neurological region and it is mediated via stimulation of classic opioid receptors (M.R. Hutchinson and S.S. Lewis *et al.*, 2007; M. R. Hutchinson, L.C. Loram *et al.*, 2010). The series of multidisciplinary studies provided converging lines of evidence that morphine binds to an accessory protein of glial toll-like receptor 4 (TLR4), myeloid differentiation protein 2 (MD-2), thereby inducing TLR4 oligomerization and triggering pro inflammation (X. Wang *et al.*, 2012). Stimulation of glial TLR4 persuades over expression of TNF- α (O. Saito *et al.*, 2010; I. Bettoni *et al.*, 2008). TNF α is one of a handful of identified gliotransmitters (M.M. Halassa and T. Fellin *et al.*, 2007; A. Volterra *et al.*, 2005). TLR4 inhibitor attenuated precipitated abolition in these morphine-dependent rats (M.R. Hutchinson *et al.*, 2010). These investigations further examined that narcotics possess abolition ailment in star shaped cells stimulation to release TNF α in the PAG and that, interestingly, exogenous TNF α injection into the PAG evokes morphine abolition like behaviors (S. Hao *et al.*, 2011). This available suggestions suggested that the investigational parameters plays a major role in the tumor necrosis factor having a chief role in the glial-neuronal interactions that influence drug abuse (P.G. Haydon *et al.*, 2009) by modulating synaptic transmission (J.S. Bains *et al.*, 2007; M. Pickering *et al.*, 2005).

PAG play vital role in the expression of many signs of opioid withdrawal. The PAG is subdivided into dorsal and ventrolateral longitudinal columns. Both the ventrolateral PAG (vlPAG) and lateral PAG (lPAG) project extensively to ventromedial and ventrolateral medullary regions (Key and Bandler *et al.*, 2004). It is rich in opioid receptors and endogenous opioid peptides and mediates physiological functions (Bandler and Shipley *et al.*, 1994 Vaughan and Christie *et al.*, 1997).

3.1.2. Hypothesis for selecting the pirfenidone and piperine for the present study

The selected drug pirfenidone (200 and 300 mg/kg; p.o) and piperine (10 and 15 mg/kg; p.o) may inhibit the neuroinflammation and brain aging. Oxidative stress in microglial cells contribute importantly in neuroinflammation. The increase in oxidative stress have been suggested to be the important processes of brain aging that cause the direct cell damage and causes opiate dependency.

An increase in oxidative stress damage associated with chronic use of opioid drug has also been shown in the neurons of the brains of human as well as rodents. It has been indicated by various studies that the damage caused by oxidative stress and increased the level of TNF- α have active contribution in pathogenesis of opioid withdrawal syndrome

Memantine have been approved clinically for opioid withdrawal syndrome. Interestingly, pirfenidone and piperine were observed for its inhibitory activity on the oxidative stress and TNF- α . Piperine also has shown an antidepressant-like activity in rodents (Landa *et al.*, 2012)

It has been reported for its antioxidant, anti-inflammatory properties for inhibiting production of oxidative stress and suppression of microglial activation and proinflammatory cytokines in the hippocampus.

Pirfenidone has been reported to attenuate neuroinflammation mediated by microglial cells through reducing activation of glial cells followed by TLR-4 and decreased the level of TNF- α . Pirfenidone has been reported to used in the treatment of various diseases like renal fibrosis, progressive multiple sclerosis, cardiac fibrosis, idiopathic pulmonary fibrosis, lung diseases and hepatic fibrosis (Salazar Montes *et al.*, 2008).

Piperine has been reported as an antiinflammatory agent against many diseases, as oxidative stress. It act as antidepressant, analgesic, antianxiety, and antioxidant. The antioxidant, anti-inflammatory effects, inhibition of proinflammation and neuroprotective effects of piperine make it very crucial, to explore piperine for evaluation against opioid withdrawal syndrome (Pradeep *et al.*, 2004).

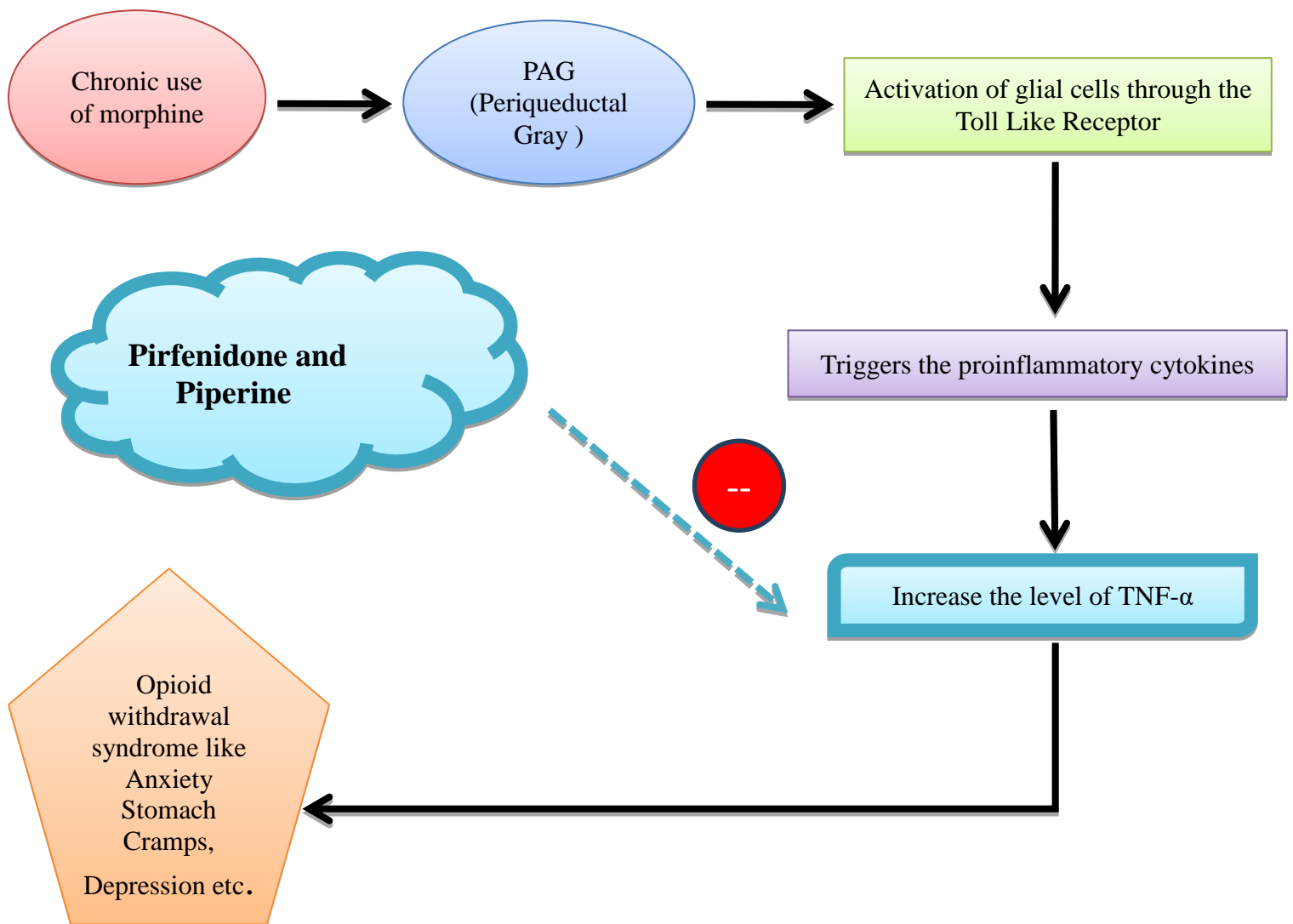


Figure 2: Flow Diagram of Hypothesis

3.2. Aim

Modulation of tumor necrosis factor- α by pirfenidone and piperine in opioid withdrawal syndrome.

3.3. Objectives

3.3.1. To induce morphine addiction in mice by administration of morphine sulphate twice daily for period of 5 days.

3.3.2. To develop morphine withdrawal syndrome by, (i) administration of naloxone on 6th day after 5 days of morphine administration in precipitated opioid withdrawal model and (ii) cessation of morphine on 6th day after 5 days morphine administration in spontaneous opioid withdrawal model.

3.3.3. To assess morphine withdrawal syndrome by evaluating various behavioral parameters and see its reversal by pirfenidone and piperine.

- Jumping frequency
- Circling frequency
- Fore paw licking
- Rearing frequency
- Withdrawal chronic score
- Anxiety effect
- Analgesic effect
- Depressant effect

3.3.4. To evaluate the effect of pirfenidone and piperine on various biochemical estimation in opioid withdrawal syndrome.

- Estimation of TBARS
- Estimation of GSH
- Estimation of SAG
- Estimation of CAT
- Estimation of TNF- α

3.3.5. To evaluate the effect of pirfenidone and piperine in vitro studies

3.4. Plan of work

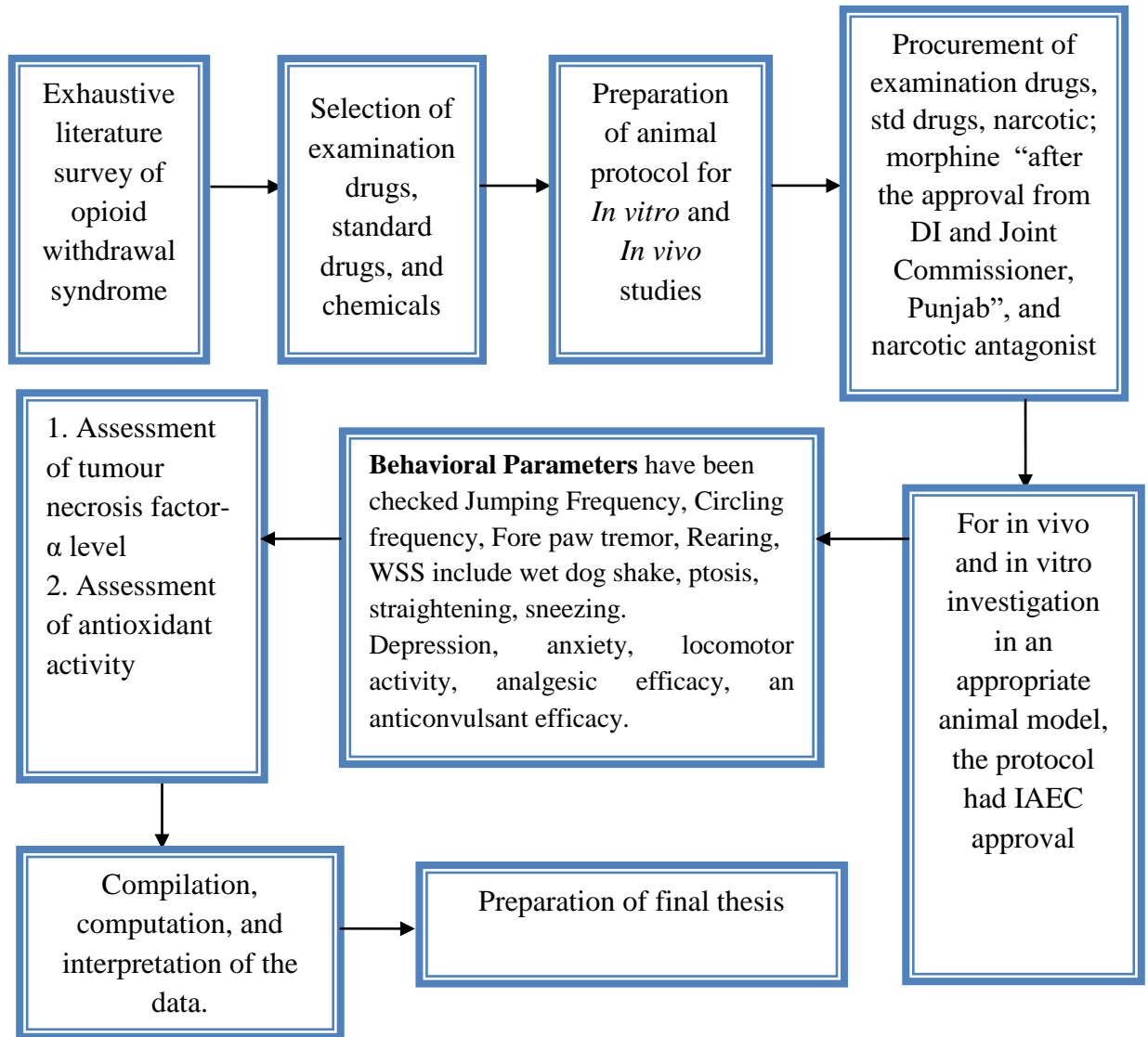


Figure 3: Flow Diagram (Plan of Work)

4. MATERIALS AND METHODS

4.1. Drugs and Chemicals

Drugs and chemicals which are used for the present research are morphine sulfate (Gupta Agencies, 2 C, Dilkusha Market, from Jalandhar, Punjab, India), Naloxone (Jackson Laboratories Ltd. Amritsar, Punjab, India), Pirfenidone (Sigma-Aldrich, St. Louis, Missouri, United States), Piperine (Sigma-Aldrich, St. Louis, Missouri, United States), Memantine (Sigma-Aldrich, St. Louis, Missouri, United States), Disodium hydrogen phosphate (LOBA Chemie Pvt. Ltd., Mumbai, India), Ethanol (Changlutathioneu Yangyuan Chemical, China), Hydrochloric acid (LOBACM), H₂O₂ (LOBACM), N-1-naphthyl ethylene diamine dihydrochloride (LOBACM), N-butanol (Central Drug House, Mumbai, India), Nitro blue tetrazolium chloride (LOBACM), Lessened glutathione (LOBACM), Sodium carbonate (Central Drug House, Mumbai, India), Sodium citamout (Central Drug House, Mumbai, India), Sodium dihydrogen phosphate (LOBACM), NaOH (Rankem, Mumbai, India), Thiobarbituric acid (LOBACM).

4.1.2. Equipments

Types of equipment used for the investigation were Activity cage (INCO Pvt. Ltd., India), Electronic weighing balance CY360 (Shimadzu Co. Ltd., Kyoto, Japan), Hot air oven, Drying Oven (Cadmach Machinery Ltd., Ahmadabad, India), Magnetic-stirrer (Remi), Perspex Identification Chamber, Elevated Plus Maze, FST, UV Spectrophotometer UV-1800 (Shimadzu Co. Ltd., Kyoto, Japan).

4.2. Animals

Animals used:

- o Species/Common name : Albino Swiss Mice
- o Age / weight / size : Adult / 20-25
- o Gender : Male and Female
- o Number of animals : 108

4.3 METHODOLOGY

4.3.1 Animals

Swiss albino mice of either sex ranging in the weight of 20-30 g each were brought from NIPER, S.A.S. Nagar, Punjab a CPCSEA registered breeding facility. Animals were transported by road by using institutional vans to avoid transport stress. Polypropylene cages of a pertinent size were provided to easy for individual animals to feel comfortable, free to move, and feel protected from every possible injury. The food and water were given in suitable containers in adequate quantity and more water during transit. They were kept in the central animal house facility at Lovely Professional University Phagwara, Punjab registered with CPCSEA vide registration number 954/PO/Re/S/06/CPCSEA. Each animal was given a 12 hour dark and 12 hour light cycle and was kept at circling humidity (60%) and temperature (26° C).

4.4. Induction of opioid addiction

4.4.1 Induction of morphine addiction in mice

Morphine was administered (5 mg/kg, i.p.) twice daily for a period of 5 days. On the sixth day, 2 h after the injection of morphine, an injection of naloxone (8 mg/kg, i.p.) was given in order to precipitate withdrawal syndrome in mice (precipitated animal model), without naloxone (spontaneous animal model). Behavioral observations were made in two phases immediately after injecting naloxone (Rehni *et al.*, 2008a, b; Rehni and Singh 2011a, 2011b; Way *et al.*, 1969). The observations were made in a transparent perspex observation chamber with dimensions of 30cm × 30cm × 30cm. One observer blind to the treatment schedule simultaneously observed each animal for all of the withdrawal measures, and the mean value of the observations was recorded. Initial 30-min observation period immediately after naloxone administration was the segment of observation period that was used to assess withdrawal severity score and jumping frequency in mice, while the second 30-min observation period (started after the completion of the earlier observation period) represented the segment of observation period that was used to assess the frequency of rearing, fore paw licking, defecation frequency, urination

frequency and circling also checked the locomotor activity, depressant activity, anxiety, analgesia. The morphine/vehicle injections were given at 6:00 a.m. and 6:00 p.m. daily. On the last day of the protocol, naloxone/vehicle was injected at 8:00 a.m. (2 h after the last morphine injection at 6:00 a.m.). However, pifenidone/piperine/combination of both/vehicle was administered at 7:00 a.m. daily until day 5.

4.5. Investigational protocol

4.5.1. Precipitated Animal model

The detailed study plan for Precipitated animal model is given below. Ten groups were used in the current investigation and each group was constituted of six Swiss albino mice. Dose and duration of the selected drugs were chosen on the basis of the literature (**Rehni *et al.*, 2012**). The dosing schedule is given in **Table 2**.

Group I:- Vehicle Control

Mice were administered with vehicle of morphine (normal saline, 5 ml/kg; i.p) twice daily for a period of 5 days (Day 1 to Day 5). Vehicle (CMC, 5 ml/kg; i.p) for pifenidone and piperine were simultaneously injected once daily for the same period of 5 days. Vehicle (normal saline 10 ml/kg; i.p) for naloxone was then injected in the morning of day 6, 2 hrs after administering vehicle for morphine.

Group II:- Pifenidone + Piperine per se treated

Mice were administered with vehicle of morphine (normal saline, 5 ml/kg; i.p) twice daily for a period of 5 days (Day 1 to Day 5). Pifenidone and piperine were simultaneously injected once daily for the same period of 5 days. On 6th day pifenidone and piperine were administered (300 mg/kg p.o and 15 mg/kg; p.o) in the morning 2 hr after administering vehicle (normal saline 10 ml/kg; i.p) for morphine.

Group III:- Morphine per se

Morphine (5 mg/kg; i.p) was administered twice daily for a period of 5 days (Day 1 to Day 5). Vehicle (CMC, 5 ml/kg; i.p) for pifenidone and piperine were simultaneously injected once daily for the same period of 5 days.

Group IV:- Morphine - Naloxone treated

Morphine (5 mg/kg; i.p) was administered twice daily for a period of 5 days (Day 1 to Day 5). Vehicle (CMC, 5 ml/kg; i.p) for pirfenidone and piperine were simultaneously injected once daily for the same period of 5 days. Naloxone (8 mg/kg; i.p) was then injected in the morning of day 6, 2 hr after administering morphine (5 mg/kg; i.p).

Group V:- Morphine - Naloxone + Memantine treatment

Morphine (5 mg/kg; i.p) was administered twice daily for a period of 5 days (Day 1 to Day 5). Memantine (10 mg/kg; i.p) was simultaneously injected once daily for the same period of 5 days. Naloxone (8 mg/kg; i.p) was then injected in the morning of day 6, 2 hr after administering morphine (5 mg/kg; i.p).

Group VI:- Morphine – Naloxone + Pirfenidone low dose

Morphine (5 mg/kg; i.p) was administered twice daily for a period of 5 days (Day 1 to Day 5). Pirfenidone (200 mg/kg; p.o) was simultaneously injected once daily for the same period of 5 days. Naloxone (8 mg/kg; i.p) was then injected in the morning of day 6, 2 hr after administering morphine (5 mg/kg; i.p).

Group VII:- Morphine – Naloxone + Pirfenidone high dose

Morphine (5 mg/kg; i.p) was administered twice daily for a period of 5 days (Day 1 to Day 5). Pirfenidone (300 mg/kg; p.o) was simultaneously injected once daily for the same period of 5 days. Naloxone (8 mg/kg; i.p) was then injected in the morning of day 6, 2 hr after administering morphine (5 mg/kg; i.p).

Group VIII:- Morphine – Naloxone + Piperine low dose

Morphine (5 mg/kg; i.p) was administered twice daily for a period of 5 days (Day 1 to Day 5). Piperine (10 mg/kg; p.o) was simultaneously injected once daily for the same mg/kg, i.p route was given in the morning of day 6, 2 h after introducing morphine 5 mg/kg via i.p route period of 5 days. Naloxone (8 mg/kg; i.p) was then injected in the morning of day 6, 2 hr after administering morphine (5 mg/kg; i.p).

Group IX:- Morphine – Naloxone + Piperine high dose

Morphine (5 mg/kg; i.p) was administered twice daily for a period of 5 days (Day 1 to Day 5). Piperine (15 mg/kg; p.o) was simultaneously injected once daily for the same period of 5 days. Naloxone (8 mg/kg; i.p) was then injected in the morning of day 6, 2 hr after administering morphine (5 mg/kg; i.p).

Group X:- Morphine – Naloxone + Pirfenidone + Piperine combination

Morphine (5 mg/kg; i.p) was administered twice daily for a period of 5 days (Day 1 to Day 5). Pirfenidone (200 mg/kg; p.o) and piperine (10 mg/kg; p.o) were simultaneously injected once daily for the same period of 5 days. Naloxone (8 mg/kg; i.p) was then injected in the morning of day 6, 2 hr after administering morphine (5 mg/kg; i.p).

Table 2. Groups for Naloxone Precipitated Opioid Withdrawal Syndrome

Sr. No.	Group name	Treatment	Amount of drug and route
1.	Control	NaCl+ 0.5 % cmc, solution	5 ml/kg i.p + 5 ml/kg p.o
2.	Pirfenidone + Piperine <i>per se</i>	NaCl+ Pirfenidone + Piperine	1 ml/kg i.p + 300 mg/kg p.o + 15 mg/kg , p.o
3.	Morphine <i>per se</i>	Morphine	5 mg/kg p.o
4.	Morphine –Naloxone	Morphine + Naloxone + 0.5 % cmc solution	5 mg/kg + 8 mg/kg i.p + 5 ml/kg p.o
5.	Morphine -Naloxone + Memantine treatment	Morphine + Naloxone+ Memantine	5 mg/kg i.p + 8 mg/kg i.p + 10 mg/kg p.o
6.	Morphine -Naloxone + Pirfenidone- low dose	Morphine + Naloxone+ Pirfenidone	5 mg/kg Intraperitoneal injection+ 8 mg/kg i.p + 200 mg/ kg p.o

7.	Morphine -Naloxone + Pirfenidone- high dose	Morphine + Naloxone+ Pirfenidone	5 mg/kg i.p + 8 mg/kg i.p + 300 mg/kg p.o
8.	Morphine -Naloxone + Piperine- low dose	Morphine + Naloxone+ Piperine	5 mg/kg i.p + 8 mg/kg i.p + 10 mg/kg p.o
9.	Morphine -Naloxone + Piperine- high dose	Morphine + Naloxone+ Piperine	5 mg/kg i.p + 8 mg/kg i.p of + 15 mg/kg p.o
10.	Morphine -Naloxone + Pirfenidone + Piperine combination	Morphine + Naloxone + Pirfenidone + Piperine	5 mg/kg i.p + 8 mg/kg i.p + 200 mg/kg p.o+ 10 mg/kg; p.o

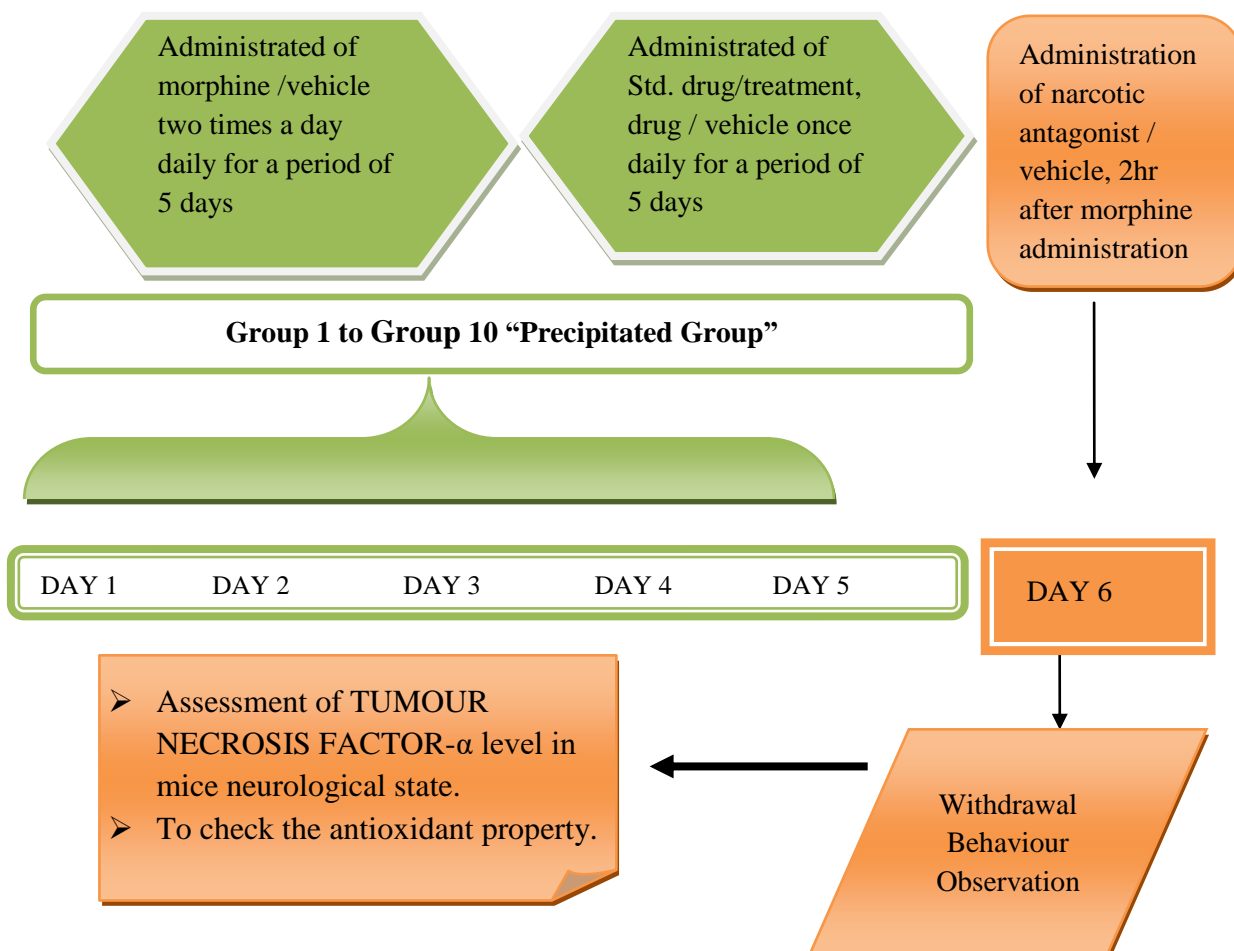


Fig. 4: Pictorial characterization of Naloxone precipitation animal model

4.5.2. Spontaneous Animal model

Morphine were administered (5 mg/kg, i.p.) two times a day for a period of 5 days. After the five days via i.p injection of morphine (5 mg/kg, i.p) was administered instead of naloxone. Behavioural observations were made for a period of 30 min immediately after the last dose of morphine to check the spontaneous behavioral response of animals. The observations were attempted in a transparent Perspex observation chamber with dimensions of 30 × 30 × 30 cm. Both examiners were blind to the study and at the same time, observed every rodent for all the observations, which was recorded data in the study.

The detailed study plan for spontaneous animal model is given below. Another ten groups were used in the recent investigation and every group consisted of six Swiss albino mice. (Rehni *et al.*, 2012). The dosing schedule is given in **Table 3**.

Group I:- Vehicle Control

Mice were administered with vehicle of morphine (normal saline, 5 ml/kg; i.p) twice daily for a period of 5 days (Day 1 to Day 5). Vehicle (CMC, 5ml/kg; i.p) for pirfenidone and piperine were simultaneously injected once daily for the same period of 5 days. Vehicle (normal saline 10 ml/kg; i.p) for naloxone was then injected in the morning of day 6, 2 hr after administering vehicle (normal saline 10 ml/kg; i.p) for morphine.

Group II:- Pirfenidone + Piperine per se

Mice were administered with vehicle of morphine (normal saline, 5 ml/kg; i.p) twice daily for a period of 5 days (Day 1 to Day 5). Pirfenidone and piperine were simultaneously injected once daily for the same period of 5 days. On 6th day, pirfenidone and piperine were administered (300 mg/kg p.o and 15 mg/kg; p.o) in the morning 2 hr after administering vehicle (normal saline 10 ml/kg; i.p) for morphine.

Group III:- Morphine per se treated

Morphine (5 mg/kg; i.p) was administered twice daily for a period of 5 days (Day 1 to Day 5). Vehicle (CMC, 5 ml/kg; i.p) for pirfenidone and piperine were simultaneously injected once daily for the same period of 5 days.

Group IV:- Morphine Withdrawal

Administration of morphine (5 mg/kg; i.p) was given two times in a day for a period of Day 1 to Day 5. On the sixth day, morphine was not administered, observed the withdrawal symptoms.

Group V:- Morphine + Memantine treatment

Morphine (5 mg/kg; i.p) was administered twice daily for a period of 5 days (Day 1 to Day 5). Memantine (10 mg/kg; i.p) was simultaneously injected once daily for the same period of 5 days.

Group VI:- Morphine + Pirfenidone low dose

Morphine (5 mg/kg; i.p) was administered twice daily for a period of 5 days (Day 1 to Day 5). Pirfenidone (200 mg/kg; p.o) was simultaneously injected once daily for the same period of 5 days.

Group VII:- Morphine + Pirfenidone high dose

Morphine (5 mg/kg; i.p) was administered twice daily for a period of 5 days (Day 1 to Day 5). Pirfenidone (300 mg/kg; p.o) was simultaneously injected once daily for the same period of 5 days.

Group VIII:- Morphine + Piperine low dose

Morphine (5 mg/kg; i.p) was administered twice daily for a period of 5 days (Day 1 to Day 5). Piperine (10 mg/kg; p.o) was simultaneously injected once daily for the same period of 5 days. Naloxone (8 mg/kg; i.p) was then injected in the morning of day 6, 2hr after administering morphine (5 mg/kg; i.p).

Group IX:- Morphine + Piperine high dose

Morphine (5 mg/kg; i.p) was administered twice daily for a period of 5 days (Day 1 to Day 5). Piperine (15 mg/kg; p.o) was simultaneously injected once daily for the same period of 5 days.

Group X:- Morphine + Pirfenidone + Piperine combination

Morphine (5 mg/kg; i.p) was administered twice daily for a period of 5 days (Day 1 to Day 5). Pirfenidone (200 mg/kg; p.o) and piperine (10 mg/kg; p.o) were simultaneously injected once daily for the same period of 5 days.

Table 3. Groups for spontaneous opioid withdrawal symptom animal model

Sr. No.	Group name	Treatment	Amount of drug and route
1.	Control	NaCl+ 0.5 %CMC solution	1 ml/kg i.p + 5 ml/kg p.o
2.	Pirfenidone+ Piperine <i>per se</i>	NaCl+ Pirfenidone + Piperine	1 ml/kg i.p + 300 mg/kg p.o + 15 mg/kg p.o
3.	Morphine <i>per se</i>	Morphine + 0.5 % CMC solution	5 mg/kg i.p + 5 ml/kg p.o
4.	Morphine Withdrawal	Morphine + 0.5 % CMC solution	5 mg/kg i.p + 5 ml/kg p.o
5.	Morphine + Memantine treatment	Morphine + Memantine	5 mg/kg i.p + 10 mg/kg p.o
6.	Morphine + Pirfenidone- low dose	Morphine + Pirfenidone	5 mg/kg i.p + 200 mg/kg p.o
7.	Morphine + Pirfenidone- high dose	Morphine + Pirfenidone	5 mg/kg i.p + 300 mg/kg p.o
8.	Morphine + Piperine- low dose	Morphine + Piperine	5 mg/kg i.p + 10 mg/kg p.o

9.	Morphine + Piperine-high dose	Morphine + Piperine	5 mg/kg i.p + 15 mg/kg p.o
10.	Morphine + Pirfenidone + Piperine combination	Morphine + Pirfenidone + Piperine	5 mg/kg i.p + 200 mg/kg + 10 mg/kg ; p.o

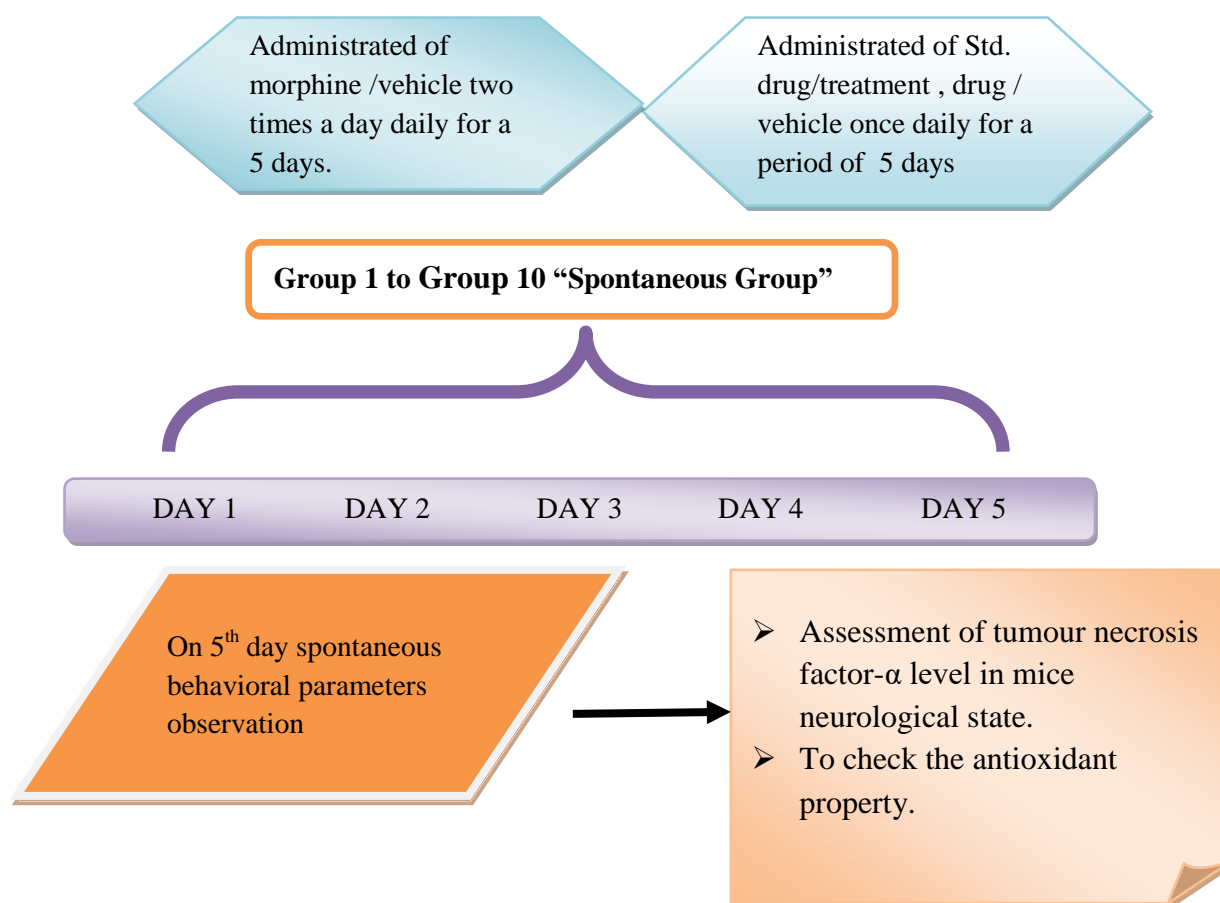


Fig 5: Pictorial characterization of spontaneous animal model

4.5.3. *In vitro* rat ileum study

The rat ileum preparation was prepared by tying a loop on one end of the tissue, and another thread was tied on a diagonally opposite aspect in order to ensure the complete opening up of the lumen of the tissue while the same was mounted in the tissue bath. The tissue was then suspended in an organ bath containing 20 ml of Krebs solution (NaCl 118, KCl 4.75, K₂HPO₄ 1.2, CaCl₂ 1.26, MgSO₄ 1.2, NaHCO₃ 25

and glucose 11.1 mM) at 37°C, aerated with 95% O₂ and 5% CO₂. A resting tension of 1 g was applied to the tissue. The tissue was allowed to equilibrate for 40–60 min, and the response to acetylcholine (ACh) was determined for three times (10⁻⁶ M) so that withdrawal response could be expressed as percentage of a particular mean ACh response. Morphine (10⁻⁶ M) was added to the bath, and the tissue was exposed to the opioid agonist for a period of 4 min. Naloxone (10⁻⁵ M) was then added in the bath to elicit a strong opioid withdrawal contracture in the rat ileum. After washout, another ACh response was obtained (to verify whether the ileum responsiveness was modified after withdrawal contracture). After 10 min of sting period, test drug/vehicle (varying concentration as per the protocol) was added in the bath along with the 4-min exposure of the ileum to the opiate [morphine (10⁻⁶ M)]. Naloxone (10⁻⁵ M) was then added to elicit a response. Following washout, ACh response was repeated to affirm the functional ability of the tissue. Moreover, in order to avoid the development of tolerance to repeated morphine exposure, each preparation was exposed only to three challenges with morphine and naloxone. Naloxone *per se* did not produce any effect on *naive* preparations or those washed after morphine contact.

4.6. Evaluation Parameters of precipitated and spontaneous animal model

4.6.1. Behavioral Parameters

4.6.1.1. Jumping frequency

Repeated jumping behavior precipitated by opioid antagonist naloxone in precipitated animal model and discontinued the morphine on 6th day in spontaneous animal model has been considered as a prominent sign for quantification of morphine withdrawal syndrome in mice. **Way *et al.*, 1969; Rehni, 2011a, b.** Jumping frequency noted in the first phase of observation period (the initial 30 min) was used as a quantitative measure of morphine withdrawal.

4.6.1.2. Withdrawal chronic score

Withdrawal severity score (WSS) was employed to quantify the withdrawal syndrome in mice in terms of earlier reported behavioral patterns, viz. fore paw tremor, wet dog shake, straightening, ptosis and sneezing, all in a composite manner

(Georgescu *et al.*, 2003; Inoue *et al.*, 2003; Liu *et al.*, 2007; Rehni *et al.*, 2008a, b; Rehni and Singh 2011a, b; Shaw- Lutchman *et al.*, 2002). In each of the individual behavioral aspect of severity scores of withdrawal, 0 score was awarded for no change in the normal behavior of mice with respect to each observation criteria, 1 score was awarded for a mild increase in the respective observation criteria, 2 score was awarded for a moderate increase in the respective observation criteria, and 3 score was awarded for a severe increase in the respective observation criteria. Thus, the higher the score is, the more severe is the withdrawal symptom. Animals were observed for each of the behavioral criteria for a period of 30 min and global score depicting both the general severity of the feature in terms of magnitude of a given episode as well as its frequency during the observation period. The cumulative withdrawal severity score was obtained by adding scores awarded to each of the five behavioral aspects, i.e. fore paw tremor, wet dog shake, straightening, ptosis and sneezing in a composite manner.

4.6.1.3. Rearing, forepaw licking, and circling frequency in mice

Rearing, fore paw licking and circling frequency observations were made during the 30-min observation period as a measure of the severity of behavioral aberration ascribed to experimental withdrawal phenomenon. These parameters have been noted to be indicative of the intensity of withdrawal syndrome and are in line with earlier reports (Glick and Morihisa 1976; Patkina and Zvartau 1978; Falls and Kelsey 1989).

4.6.1.4. Locomotor activity

Locomotor activity was evaluated using an activity cage (called as actophotometer) an activity cage consisting of a square metallic chamber with infrared photocell beams located across the frame axis. Crossing of the beam by the mice was taken as one count and the number of counts in 10 minutes was recorded for individual rats (Khurana *et al.*, 2011).

4.6.1.5. Evaluation of the analgesic efficacy

Nociceptive threshold was measured by the tail flick test in mice (D'Amour and Smith 1941). The tail flick latency was considered as the time between tail exposure to radiant heat and tail withdrawal. Electrically heated nichrome wire was used as a source of radiant heat in the analgesiometer. The intensity of radiant heat was regulated in order to obtain pretreatment latency between 2 and 3s in the animals. The cut-off latency time was fixed at 10 s. Tail flick latency was expressed as a percentage of the maximum possible withdrawal behavior of the animal. It served as a measure of analgesic activity.

(MPE)

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

4.6.1.6. Evaluation of antidepressant activity

In the **forced swim test** mice was placed in a small, confined space, such as a large graduated cylinder filled halfway with water. Initially, there is a period of vigorous activity during which the mice tries to escape. Eventually, the animal ceases vigorous activity and exhibits a characteristic immobility in which it only moves to maintain its head above water. This physical immobility is thought to be an indication of behavioral despair. Investigator measure the amount of time between when the animal is placed in the chamber and the onset of immobility (Yankelevitch-Yahav et al., 2015).

First day and on the last day of the examination 6th day, the succeeding conduct responses were noted. Fixed status time enjoyed drifting with less developments to keep the head over the water; and swimming time enjoyed with dynamic swimming developments. It filled in as an assurance of the energizer movement.

4.6.1.7. Assessment of antianxiety activity

Instrument (Elevated Plus Maze) contains four arms that are raised 50 cm over the floor, having the two enclosed arms $16 \times 5 \times 12$ cm with an open roof top and two open arms with the components of 16×15 cm and. Assessments were made in a sound free room.

On day 1 and day 6, the following parameters were observed for five minutes:

1. Time spent in open arm
2. No. of entries in open arm.

4.6.2. Biochemical Estimations

After the completion of behavioral studies on the last day of treatment, animals marked for biochemical studies were sacrificed by cervical dislocation. The brains were removed, weighed and divided into two sets. One set of brains were triturated to make a 10% (w/v) tissue homogenate in 0.1 M phosphate buffer (ph 7.4). The clear supernatant obtained after centrifugation at 3000 rpm for 15 minutes, was used for various biochemical studies to estimate the levels of TNF- α , TBARS, GSH, CAT. Another set of brains were used for the estimation of SAG.

4.6.2.1. Assessment of tumor necrosis factor- α level in mice brain

Neurological phases of tumour necrosis factor-a, IL-6, IL-1 β , and IL-10 were assessed by chemical immunoassay packs as expounded in the past (de Waal Malefyt *et al.*, 1991). Measures were recognized and done by means of producer's rules Bioscience, Vienna, Austria. Shading that invulnerabilities that not set in stone at 450 nm through the usage of a microplate peruser.

4.6.2.2. Assessment of thiobarbituric destructive responsive substances (TBARS)

The quantitative assessment of thiobarbituric destructive responsive substances is a trace of lipid peroxidation in the neurological state. It was executed by the methodology of Ohkawa *et al.*, 1979.

Preparation of reagents

Preparation of sodium dodecyl sulphate solution:

Sodium dodecyl sulphate (810 mg) was dissolved in distilled water (10 mL).

Preparation of 30% acetic acid solution:

Acetic acid (30 mL) was diluted to 100 mL with distilled water and pH was adjusted to 3.5 with saturated solution of sodium hydroxide using pH meter.

Preparation of 0.8% thiobarbituric acid solution:

Thiobarbituric acid (400 mg) was dissolved in warm distill water (50 mL).

Preparation of 15:1 v/v n-butanol-pyridine mixture:

n-butanol (90 mL) was mixed with pyridine (6 mL).

Preparation of 1 nM 1, 3, 3-tetramethoxy propane:

1, 1, 3, 3-tetramethoxy propane (0.82 mL) was diluted to 5 mL with distilled water to make 1 M solution. 1 mL of above solution was further diluted to 10 mL with distilled water and this dilution process was further repeated eight times to get 1nM 1, 1, 3, 3-tetramethoxy propane.

Procedure

Supernatant of homogenate (0.2 mL) was pipetted out in a test tube, followed by addition of sodium dodecyl sulphate (8.1%; 0.2 mL), acetic acid (30%; 1.5 mL; pH 3.5), thiobarbituric acid (1.5 mL) and the volume was made up to 4 mL with distill water. The test tubes were incubated for 1 h at 95 temp, cooled and distill water (1 mL) was added followed by addition of *n*-butanol-pyridine mixture (15:1 v/v; 5 mL). The tubes were centrifuged at 4000 g for 10 min. the absorbance of the developed pink color was measured spectrophotometrically (Shimadzu UV spectrophotometer 1240) at 532 nm. The TBARS value was expressed as nanomoles per mg of protein.

4.6.2.3. Estimation of GSH

The GSH content in tissue was estimated using the method of **Beutler *et al.*, 1963.**

Preparation of reagents

Preparation of 10% trichloroacetic acid:

Trichloroacetic acid (10 g) was dissolved in distilled water (100 mL).

Preparation of 0.3 M disodium hydrogen phosphate:

Anhydrous disodium hydrogen phosphate (4.26 g) was dissolved in distilled water (100 mL).

Preparation of 5,5-dithiobis (2-nitrobenzoic acid) in 1% sodium citrate:

5,5-dithiobis [2-nitrobenzoic acid] (7.92 mg) was dissolved in sodium citrate (1%; 20 mL).

Preparation of 100 μ M of reduced glutathione:

Reduced glutathione (6.14 mg) was dissolved in distilled water (200 mL).

Procedure

The supernatant of homogenate was mixed with trichloroacetic acid (10% w/v) in 1:1 ratio. The tubes were centrifuged at 1000 g for 10 min at 4° C. The supernatant (0.5 mL) obtained was mixed with disodium hydrogen phosphate (0.3 M; 2 mL). Then freshly prepared DTNB [5, 5-dithiobis (2-nitrobenzoic acid) dissolved in 1% w/v sodium citrate; 0.001 M; 0.25 mL] was added and absorbance was noted spectrophotometrically at 412 nm. A standard curve was plotted using 10-100 μ M of reduced form of glutathione and results were expressed in micromoles of reduced glutathione per mg of protein.

4.6.2.4. Estimation of CAT

The CAT activity was estimated using method of **Aebi, 1974**.

Preparation of reagents

Preparation of 50mM phosphate buffer

Potassium dihydrogen phosphate (680 mg) was dissolved in distilled water (100 mL). Sodium hydroxide (200 mg) was dissolved in distilled water (100 mL). Above potassium dihydrogen phosphate solution (50 mL) was mixed with sodium hydroxide solution (29.1 mL) to give phosphate buffer (50 mM).

Preparation of 30 mM hydrogen peroxide

Hydrogen peroxide (0.30 mL) was added in water (up to 100 mL).

Procedure

The supernatant (50 μ l) was added to a 3.0 mL cuvette that contained phosphate buffer (50 mM; 1.95 mL; pH 7.0). Hydrogen peroxide (30 mM; 1.0 mL) was added and changes in absorbance were observed at 15 sec intervals for 30 sec at 240 nm. The catalase activity was calculated using the millimolar extinction coefficient of H₂O₂ (0.071 mmol cm) and the activity was expressed as micromoles of H₂O₂ oxidized per minute per milligram protein (**Bisswanger, 2004**).

4.6.2.5. Superoxide anion generation assay SAG

The SAG was estimated by using the reduced nitrobluetetrazolium (NBT) method of **Wang et al. (1998)**.

Preparation of reagents

Preparation of phosphate buffered saline:

Sodium hydroxide (8 g), potassium hydroxide (0.20 g), disodium hydrogen phosphate (1.44 g) and potassium dihydrogen phosphate

Preparation of NBT (100 μ M):

Nitrobluetetrazolium (8.176 g) was dissolved in distilled water (100 mL).

Preparation of 0.5 M HCl:

Conc. HCl (4.42 ml) was slowly added in distilled water (100 mL).

Preparation of 0.1 M sodium hydroxide:

Sodium hydroxide (0.4 g) was dissolved in distilled water (100 mL).

Preparation of 0.1% sodium dodecyl sulphate:

SDS (0.1 g) was dissolved in distilled water (100 mL).

Preparation of 40 mg/L diethylenetriaminepenta acetic acid:

Diethylenetriaminepenta acetic acid (40 mg) was dissolved in distilled water (1000 mL).

Procedure

A weighed amount of brain tissue (25 mg) was taken in phosphate buffered saline (5 mL) containing NBT (100 μ M) and incubated at 37°C for 1.5 h. The NBT reduction was stopped by adding HCl (0.5 M; 5 mL). The tissue was minced and homogenized in a 10% w/v mixture of sodium hydroxide (0.1 M) and sodium dodecyl sulphate (0.1%) containing diethylenetriaminepenta acetic acid (40 mg/L). The mixture was centrifuged at 20,000 g for 20 min and the resultant pellet was suspended in pyridine (1.5 mL) and kept at 80° C for 1.5 h to extract formazan, an adduct formed after reaction of NBT with superoxide anions. The mixture was again centrifuged at 10,000 g for 10 min and absorbance of formazan was determined spectrophotometrically at 540 nm.

4.7. Statistical analysis

All the results were given as mean \pm SEM. The data analysis was done by Sigma Stat Software, 4.0 using one-way analysis of variance (ANOVA) following Tukey test. $P < 0.05$ values were considered for statistical significance for all comparisons.

5. RESULTS AND DISCUSSION

5.1. Results

5.1.1. Behavioural evaluations of precipitated animal model

5.1.1.1. Effect of pirfenidone and piperine on jumping frequency

Administration of pirfenidone + piperine *per se* to group 2 and morphine *per se* to group 3 for 5 consecutive days did not show any significant change in mice jumping frequency, as compared to vehicle treated control group (group 1). Administration of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days followed by a single injection of naloxone (8 mg/kg; i.p.) 2 hours after morphine injection to group 4 precipitated the withdrawal syndrome in mice, as reflected by a significant ($p < 0.001$) increase in jumping behavioral of mice, when compared to morphine *per se* treated group 3.

The jumping behavior seen due to precipitation of naloxone- induced morphine withdrawal symptoms were significantly ($p < 0.001$) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine- naloxone treated group. The best effect was observed in pirfenidone (300 mg/kg), piperine (15 mg/kg; p.o) and combination treated groups which was significantly ($p < 0.001$) better than the standard memantine treated group. Thus, this combination treatment attenuated the morphine withdrawal symptoms as depicted by reduced jumping behavior in morphine dependent mice.

Table 4: Effect of pirfenidone and piperine on jumping frequency

Group no.	Group name	Jumping frequency Mean \pm SD
1.	Control	1.50 \pm 0.408
2.	Pirfenidone + Piperine	2.50 \pm 0.516

3.	Morphine <i>per se</i>	2.16±0.98
4.	Morphine + Naloxone	19.83±2.04 ^{***}
5.	Morphine + Naloxone+ Memantine	14.00±1.26 ^{###}
6.	Morphine + Naloxone+ Pirfenidone dose	12.66±0.51 ^{###}
7.	Morphine + Naloxone+ Pirfenidone high dose	9.00±1.09 ^{###, ^^}
8.	Morphine + Naloxone+ Piperine low dose	13.16±1.94 ^{###}
9.	Morphine + Naloxone+ Piperine high dose	9.66±1.63 ^{###, ^^}
10.	Morphine + Naloxone+ Pirfenidone + dose	7.83±0.408 ^{###, ^^}

* p<0.05, ** p<0.01, *** p<0.001 reveals remarkable variation when group 1 versus with group 2, 3 and 4, #p<0.05, ##p<0.01, ###p<0.001 reveals remarkable variation when group 4 distinguish with divisions 5, 6, 7, 8, 9, 10; ^p<0.05, ^^p<0.01, ^^p<0.001 reveals remarkable variation when group5 compared with divisions 6,7,8, 9, 10.

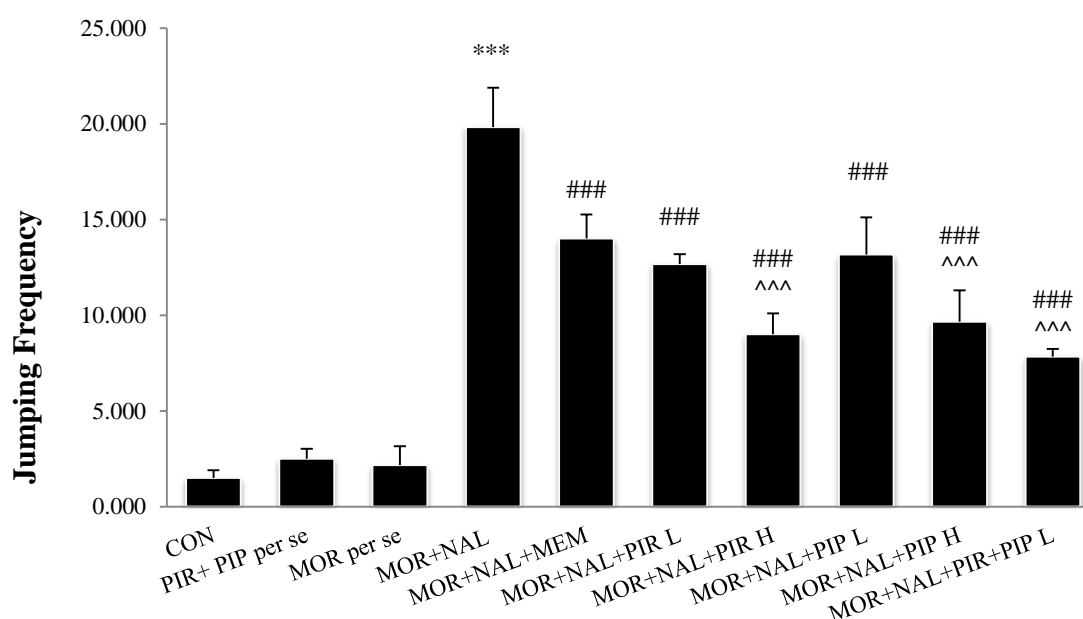


Fig. 6: Effect of pirfenidone and piperine on jumping frequency

Data represented as mean ± SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine ; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose.

* p<0.05, ** p<0.01, *** p<0.001 reveals remarkable variation when group 1 versus with

group 2, 3 and 4, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ reveals remarkable variation when group 4 distinguish with group 5, 6, 7, 8, 9, 10; ^ $p < 0.05$, ^^ $p < 0.01$, ^^ $p < 0.001$ reveals remarkable alterations when group 5 versus divisions 6, 7, 8, 9, 10.

5.1.1.2. Effect of pirfenidone and piperine on circling frequency

There was no change in mice circling frequency when administration of pirfenidone + piperine to pirfenidone piperine *per se* group in mice and morphine *per se* to morphine *per se* group in mice for 5 consecutive as compared to vehicle treated control group. Administration of morphine (5mg/kg; i.p.) twice daily for a period of 5 days followed by a single injection of naloxone (8mg/kg; i.p.) 2 hours after morphine injection to morphine naloxone group in mice precipitated the withdrawal syndrome, as reflected by a significant ($p < 0.001$) increase in circling behavioral of mice, when compared to morphine *per se* treated group.

The stereotyped circling behavior seen due to precipitation of naloxone-induced morphine withdrawal symptoms were significantly ($p < 0.001$) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine-naloxone treated group.. The best effect was observed in pirfenidone (200 mg/kg; p.o) ($p < 0.05$) and combination treated groups which was significantly ($p < 0.001$) better than the standard memantine treated group. Thus, the combination (pirfenidone 200 mg/kg; p.o and piperine 10 mg/kg; p.o) treatment attenuated the morphine withdrawal symptoms as depicted by reduced circling behavior in morphine dependent mice.

Table 5: Effect of pirfenidone and piperine on circling frequency

Group no.	Group name	Circling frequency Mean \pm SD
1.	Control	4.66 \pm 0.51
2.	Pirfenidone + Piperine	3.83 \pm 0.75

3.	Morphine <i>per se</i>	4.66±0.51
4.	Morphine + Naloxone	22.00±2.53 ^{***}
5.	Morphine + Naloxone+ Memantine	15.16±1.47 ^{###}
6.	Morphine + Naloxone+ Pirfenidone low dose	13.00±2.09 ^{###^}
7.	Morphine + Naloxone+ Pirfenidone high dose	12.00±1.54 ^{###}
8.	Morphine + Naloxone+ Piperine low dose	13.33±2.50 ^{###}
9.	Morphine + Naloxone+ Piperine high dose	12.16±1.16 ^{###}
10.	Morphine + Naloxone+ Pirfenidone + Piperine low dose	7.83±1.47 ^{###, ^^}

^{***} p<0.001 reveals remarkable variation when group 1 distinguish with division 2, 3 and 4, ^{###} p<0.001 reveals remarkable variation when group 4 versus divisions 5, 6, 7, 8, 9, 10; [^] p<0.05, ^{^^} p<0.001 reveals remarkable alterations when division 5 versus with group 6, 7, 8, 9, 10.

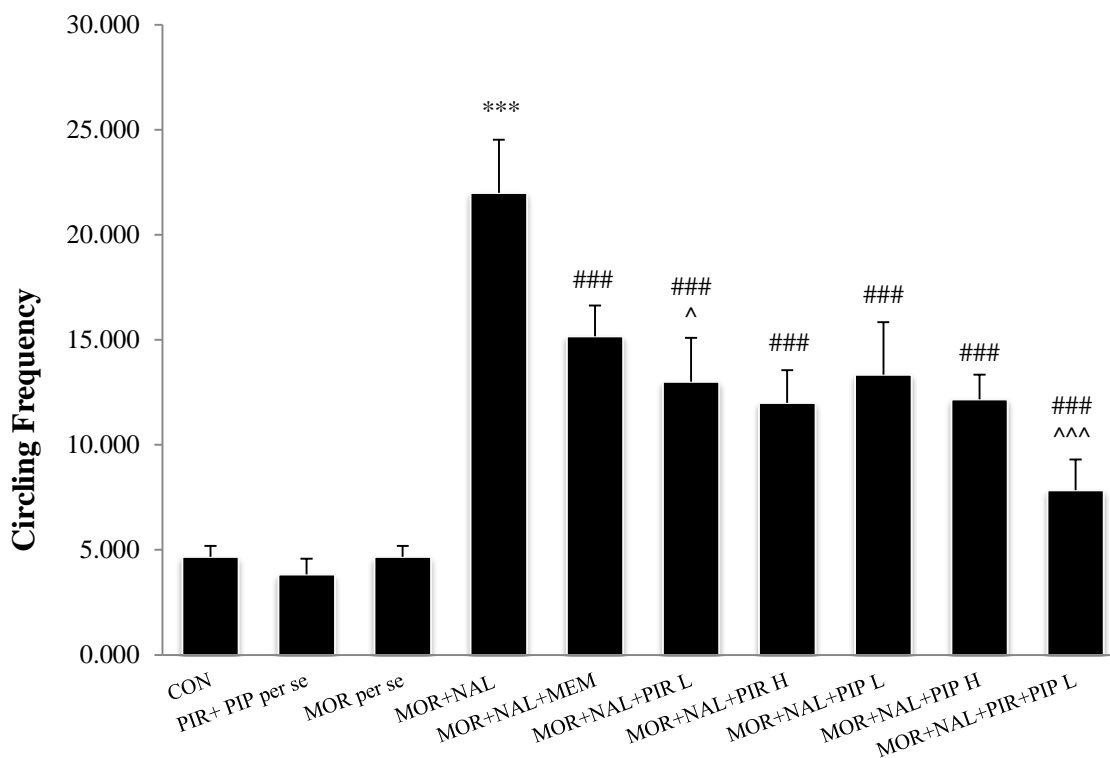


Fig. 7: Effect of pirfenidone and piperine on circling frequency

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine ; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. *** $p < 0.001$ reveals remarkable variation when group 1 distinguished along divisions 2, 3 and 4, #### $p < 0.001$ reveals remarkable variation when group 4 distinguished along divisions 5, 6, 7, 8, 9, 10; ^ $p < 0.05$, ^^ $p < 0.001$ reveals remarkable variation when division 5 versus with group 6, 7, 8, 9, 10.

5.1.1.3. Effect of pirfenidone and piperine on rearing frequency

It was observed that administration of pirfenidone + piperine to pirfenidone-piperine group in mice and morphine *per se* to morphine *per se* group in mice for 5 consecutive days did not showed any significant change in mice rearing frequency, as compared to vehicle treated control group. Administration of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days followed by a single injection of naloxone (8 mg/kg; i.p.) 2 hours after morphine injection to morphine naloxone group in mice precipitated the withdrawal syndrome, as reflected by a significant ($p < 0.001$) increase in rearing behavioral of mice, when compared to morphine *per se* treated group.

The stereotyped rearing behavior seen due to precipitation of naloxone-induced morphine withdrawal symptoms were significantly ($p < 0.001$) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine-naloxone treated group. The best effect was reported in pirfenidone (300 mg/kg; p.o) ($p < 0.01$), piperine (15 mg/kg; p.o) ($p < 0.01$) and combination treated groups which was significantly ($p < 0.001$) better than the standard memantine treated group. Thus, this combination treatment attenuated the morphine withdrawal symptoms as depicted by reduced rearing behavior in morphine dependent mice.

Table 6: Effect of pirfenidone and piperine on Rearing frequency

Group no.	Group name	Rearing frequency Mean \pm SD
1.	Control	1.66 \pm 0.81
2.	Pirfenidone + Piperine	4.33 \pm 0.51
3.	Morphine <i>per se</i>	1.83 \pm 0.75
4.	Morphine + Naloxone	33.33 \pm 0.51 ^{***}
5.	Morphine + Naloxone+ Memantine	18.66 \pm 2.50 ^{###}
6.	Morphine + Naloxone+ Pirfenidone low dose	16.66 \pm 2.25 ^{###}
7.	Morphine + Naloxone+ Pirfenidone high dose	13.50 \pm 2.34 ^{###, ^^}
8.	Morphine + Naloxone+ Piperine low dose	15.16 \pm 1.72 ^{###}
9.	Morphine + Naloxone+ Piperine high dose	13.83 \pm 2.78 ^{###, ^^}
10.	Morphine + Naloxone+ Pirfenidone + Piperine low dose	11.33 \pm 2.73 ^{###, ^^}

^{***} p<0.001 reveals remarkable variation when group 1 versus group 2, 3 and 4,

^{###} p<0.001 reveals remarkable variation when division 4 versus with group 5, 6, 7, 8,

9, 10; [^] p<0.05, ^{^^} p<0.001 reveals remarkable variation when group 5 versus with group 6, 7, 8, 9, 10.

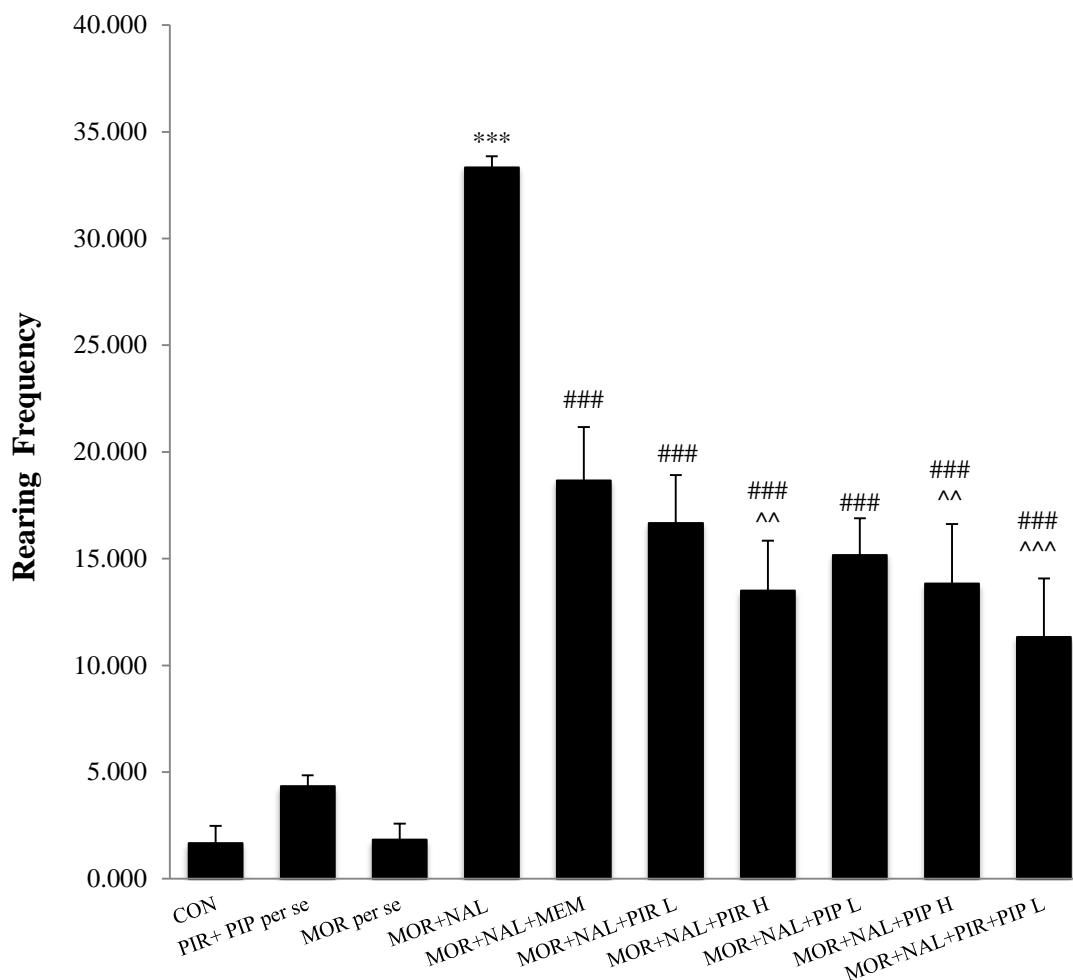


Fig. 8: Effect of pirfenidone and piperine on rearing frequency

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine ; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. *** $p < 0.001$ reveals notably variation of group 3 vs group 4; ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^^ $p < 0.01$, ^^^ $p < 0.001$ represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.1.4. Effect of pirfenidone and piperine on forepaw licking frequency

Administration of morphine *per se* to morphine *per se* group and pirfenidone + piperine to pirfenidone-piperine *per se* group mice for 5 consecutive days did not show any significant change in mice fore paw licking frequency, as compared to vehicle treated control group. But when administration of morphine

(5 mg/kg; i.p.) twice daily for a period of 5 days followed by a single injection of naloxone (8 mg/kg; i.p.) 2 hours after morphine injection to morphine naloxone group mice precipitated the withdrawal syndrome, as reflected by a significant ($p < 0.001$) increase in fore paw licking behavioral of mice, when compared to morphine *per se* treated group.

The stereotyped fore paw licking behavior seen due to precipitation of naloxone-induced morphine withdrawal symptoms were significantly ($p < 0.001$) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pifenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pifenidone-200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine-naloxone treated group. The best effect was observed in piperine (15 mg/kg) ($p < 0.01$) and combination treated groups which was significantly ($p < 0.001$) better than the standard memantine treated group. Thus, this combination treatment attenuated the morphine withdrawal symptoms as depicted by reduced fore paw licking behavior in morphine dependent mice.

Table 7: Effect of pifenidone and piperine on forepaw licking frequency

Group no.	Group name	Fore paw licking frequency Mean \pm SD
1.	Control	1.66 \pm 0.81
2.	Pirfenidone + Piperine	2.83 \pm 0.40
3.	Morphine <i>per se</i>	1.5 \pm 1.048
4.	Morphine + Naloxone	11.33 \pm 0.51 ^{***}
5.	Morphine + Naloxone+ Memantine	6.16 \pm 0.98 ^{###}
6.	Morphine + Naloxone+ Pirfenidone low dose	7.33 \pm 0.81 ^{###}
7.	Morphine + Naloxone+ Pirfenidone high dose	6.33 \pm 0.81 ^{###}
8.	Morphine + Naloxone+ Piperine low dose	7.66 \pm 0.51 ^{###}
9.	Morphine + Naloxone+ Piperine high dose	4.50 \pm 0.83 ^{###, ^^}

10.	Morphine + Naloxone+ Pirfenidone + Piperine low dose	3.16±0.98 ^{###, ^^}
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^{***}p<0.001 represent significant difference ingroup 3 vs 4; ^{###}p<0.001represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^{^^}p<0.01, ^{^^^}p<0.001 represent significant difference ingroup 5 vs 6, 7, 8, 9, 10 i.e. one-way ANALYSIS OF VARIANCE cconsective by Tukey's various prominent examination.

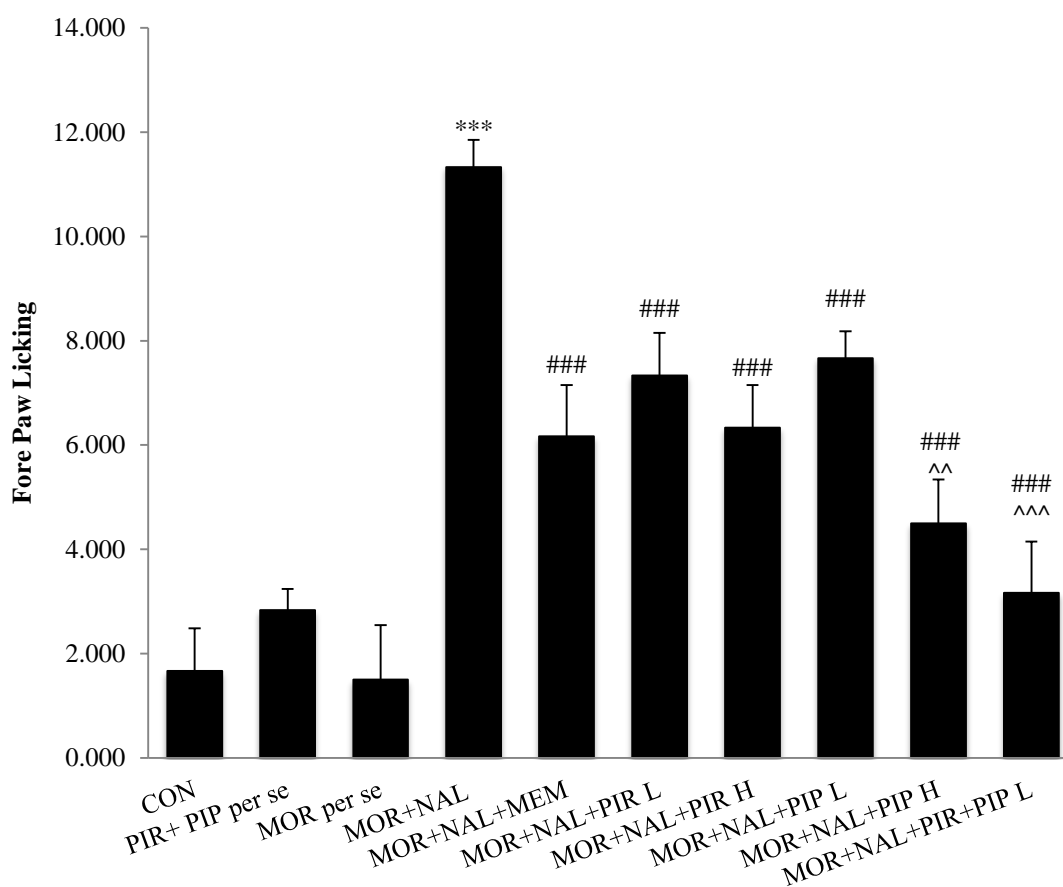


Fig. 9: Effect of pirfenidone and piperine on fore paw licking frequency

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose.

^{***}p<0.001 represent significant difference ingroup 3 vs division 4; #p<0.05, ##p<0.01, ###p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^{^^}p<0.01, ^{^^^}p<0.001 represent significant difference ingroup 5 vs 6, 7, 8, 9, 10.

5.1.1.5. Effect of pirfenidone and piperine on withdrawal chronic score WSS

Administration of morphine *per se* to morphine *per se* group and pirfenidone + piperine to pirfenidone piperine *per se* group mice for 5 consecutive days did not showed any significant change in mice withdrawal severity score (fore paw tremor, wet dog shake, ptosis and sneezing) frequency, as compared to vehicle treated control group. But when administration of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days followed by a single injection of naloxone (8 mg/kg; i.p.) 2 hours after morphine injection to morphine maloxone group mice precipitated the withdrawal syndrome, as reflected by a significant ($p < 0.001$) increase in withdrawal severity score behavioral of mice, when compared to morphine *per se* treated group.

The stereotyped WSS behavior seen due to precipitation of naloxone-induced morphine withdrawal symptoms were significantly ($p < 0.001$) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine-naloxone treated group. The best effective treatment were observed in pirfenidone (300 mg/kg; p.o) ($p < 0.001$) piperine (15 mg/kg) ($p < 0.001$) (10 mg/kg; p.o) ($p < 0.01$) and combination treated groups (pirfenidone 200 mg/kg; p.o, piperine 10 mg/kg; p.o) which was significantly ($p < 0.001$) better than the standard memantine treated group. Thus, the combination of both the treatment drugs attenuated the morphine withdrawal symptoms as depicted by reduced WSS behavior in morphine dependent mice

Table 8: Effect of pirfenidone and piperine on withdrawal chronic score WSS

Group no.	Group name	WSS frequency Mean \pm SD
1.	Control	2.33 \pm 1.03
2.	Pirfenidone + Piperine	2.50 \pm 0.54
3.	Morphine <i>per se</i>	1.33 \pm 0.81
4.	Morphine + Naloxone	15.33 \pm 0.51***

5.	Morphine + Naloxone+ Memantine	9.33±0.51 ^{###}
6.	Morphine + Naloxone+ Pirfenidone low dose	8.33±1.21 ^{###}
7.	Morphine + Naloxone+ Pirfenidone high dose	5.83±1.16 ^{###,^^}
8.	Morphine + Naloxone+ Piperine low dose	7.00±1.54 ^{###,^^}
9.	Morphine + Naloxone+ Piperine high dose	5.16±0.40 ^{###,^^}
10.	Morphine + Naloxone+ Pirfenidone + Piperine low dose	3.83±0.40 ^{###,^^}

^{***}p<0.001 reveals notably variation of group 3 vs group 4; ^{###}p<0.001 represent significant difference group 4 vs group 5, 6, 7, 8, 9, 10; ^{^^}p<0.01, ^{^^^}p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way ANALYSIS OF VARIANCE come succed by Tukey's comparison test.

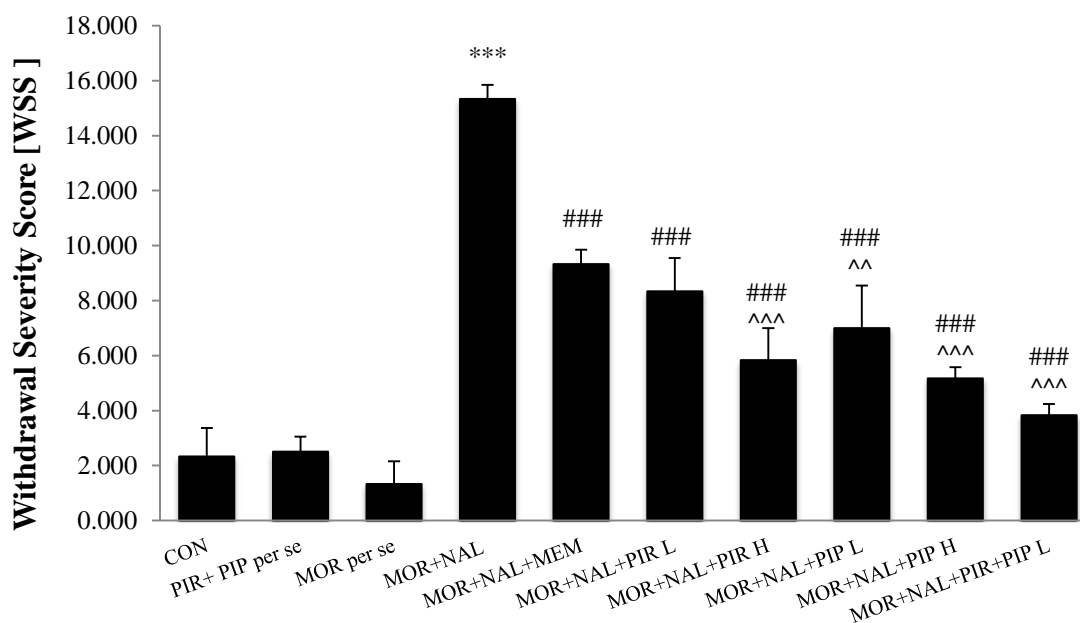


Fig. 10: Effect of pirfenidone and piperine on withdrawal chronic score

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine ; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. ^{***}p<0.001 represent significant difference ingroup 3vs group 4; #p<0.05, ##p<0.01, ###p<0.001 represent significant difference ingroup 4 vs group 5, 6, 7, 8, 9, 10; ^{^^}p<0.01, ^{^^^}p<0.001 represent significant difference ingroup 5 vs 6, 7, 8, 9, 10.

5.1.1.6. Effect of pirfenidone and piperine on Locomotor activity

There was no change in locomotor activity, when administration of morphine *per se* to morphine *per se* group and pirfenidone + piperine to pirfenidone piperine *per se* group in mice for 5 consecutive days in mice as compared to vehicle treated control group. But when administration of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days followed by a single injection of naloxone (8 mg/kg; i.p.) 2 hours after morphine injection to group 4 mice precipitated the withdrawal syndrome, as reflected by a significant ($p < 0.001$) decrease in locomotion behavioral of mice, when compared to morphine *per se* treated group.

The locomotion behavior seen due to precipitation of naloxone-induced morphine withdrawal symptoms were significantly and dose dependently increased the counts of locomotion in memantine (10 mg/kg; p.o.) ($p < 0.01$) pirfenidone (200 and 300 mg/kg; p.o.) ($p < 0.001$), piperine (10 and 15 mg/kg; p.o.) ($p < 0.001$) and combination (pirfenidone 200 mg/kg + piperine-0 10 mg/kg; p.o.) ($p < 0.001$) treated groups, when compared to morphine-naloxone treated group. The supreme effect was observed in pirfenidone (300 mg/kg; p.o.) ($p < 0.05$), piperine (15 mg/kg) ($p < 0.01$) and combination treated groups which was significantly ($p < 0.001$) better than the standard memantine treated group. Thus, this combination treatment attenuated the morphine withdrawal symptoms as depicted by increased the no. of counts in locomotion behavior in morphine dependent mice.

Table 9: Effect of pirfenidone and piperine on Locomotor activity

Group no.	Group name	Counts Mean \pm SD
1.	Control	394.33 \pm 41.21
2.	Pirfenidone + Piperine	390.66 \pm 34.13
3.	Morphine <i>per se</i>	380.5 \pm 44.84
4.	Morphine + Naloxone	210.66 \pm 11.86 ^{***}
5.	Morphine + Naloxone+ Memantine	302.33 \pm 36.65 ^{##}

6.	Morphine + Naloxone+ Pirfenidone low dose	331.33±31.63 ^{###}
7.	Morphine + Naloxone+ Pirfenidone high dose	375.50±33.76 ^{###,^}
8.	Morphine + Naloxone+ Piperine low dose	341.16±34.82 ^{###}
9.	Morphine + Naloxone+ Piperine high dose	379.50±36.51 ^{###, ^^}
10.	Morphine + Naloxone+ Pirfenidone + Piperine low dose	413.16±40.71 ^{###, ^^}

^{***}p<0.001 reveals notably variation of group 3 vs division 4, ^{###}p<0.001 reveals main variation in group 4 vs group 5, 6, 7, 8, 9, 10; [^]p<0.05, ^{^^}p<0.01, ^{^^^}p<0.001 represent significant difference ingroup 5vs 6, 7, 8, 9, 10 one-way ANALYSIS OF VARIANCE come succed by Tukey's comparsion test.

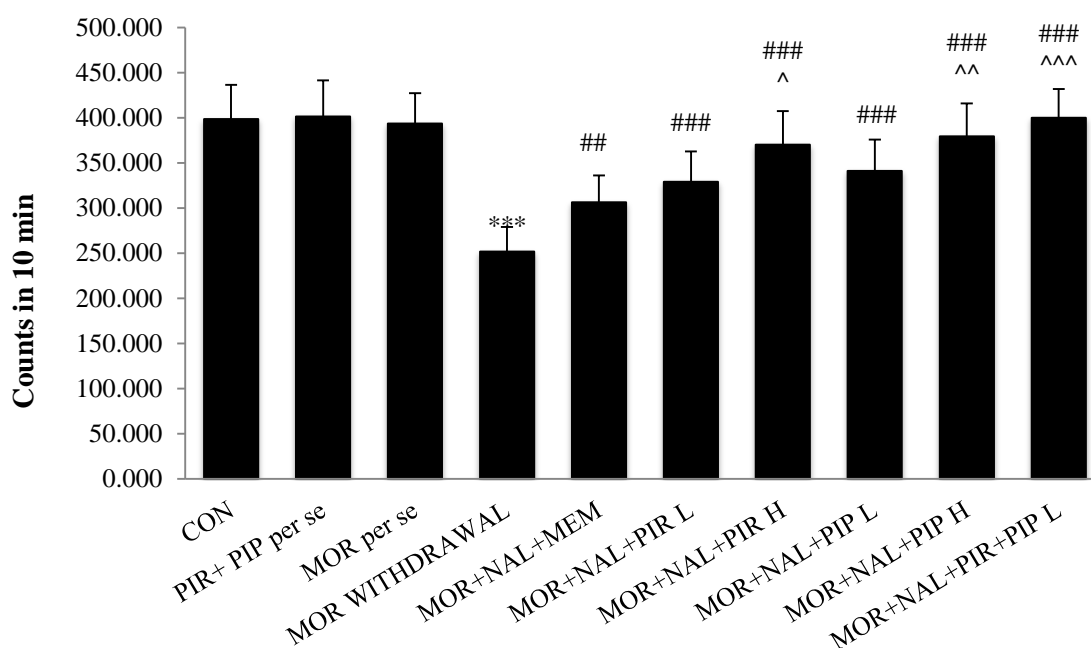


Fig. 11: Effect of pirfenidone and piperine on Locomotoractivity

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine ; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. ^{***}p<0.001 represent significant difference ingroup 3 vs division 4; ^{##}p<0.01, ^{###}p<0.001 represent significant difference of group 4 vs group 5, 6, 7, 8, 9, 10; [^]p<0.05, ^{^^}p<0.01, ^{^^^}p<0.001 represent significant difference ingroup 5 vs 6, 7, 8, 9, 10.

5.1.1.7. Effect of pirfenidone and piperine on depressant activity

One way ANOVA of the total immobility time in mice on day 6 revealed that no significant change in mice when administration of morphine *per se* to morphine *per se* group and pirfenidone + piperine to pirfenidone piperine *per se* group in mice for 5 consecutive days for depressant behavior as withdrawal syndrome in mice, as compared to vehicle treated control group. But when administration of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days followed by a single injection of naloxone (8 mg/kg; i.p.) 2 hours after morphine injection to group 4 mice precipitated the withdrawal syndrome, as reflected by a significant ($p < 0.001$) increase in immobility time, when compared to morphine *per se* treated group.

The depressant behavior seen due to precipitation of naloxone-induced morphine withdrawal symptoms were significantly ($p < 0.001$) and dose dependently increased in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine-naloxone treated group. The good effect was observed in pirfenidone (300 mg/kg; p.o) ($p < 0.05$), piperine (15 mg/kg; p.o.) ($p < 0.01$) and combination treated groups which was significantly ($p < 0.001$) better than the standard memantine treated group. Thus, the combination treatment attenuated the morphine withdrawal symptoms as depicted by reduced the immobility time (depressant behavior) in morphine dependent mice.

Table 10: Effect of pirfenidone and piperine on Depressant activity

Group no.	Group name	Immobility time Mean \pm SD
1.	Control	128.16 \pm 13.04
2.	Pirfenidone + Piperine	128.33 \pm 12.14
3.	Morphine <i>per se</i>	129.66 \pm 13.72
4.	Morphine + Naloxone	243.00 \pm 25.58 ^{***}

5.	Morphine + Naloxone+ Memantine	186.66±18.92 ^{###}
6.	Morphine + Naloxone+ Pirfenidone low dose	160.50±15.68 ^{###}
7.	Morphine + Naloxone+ Pirfenidone high dose	155.66±15.04 ^{###,^}
8.	Morphine + Naloxone+ Piperine low dose	157.83±15.69 ^{###}
9.	Morphine + Naloxone+ Piperine high dose	151.50±14.50 ^{###,^^}
10.	Morphine + Naloxone+ Pirfenidone + Piperine low dose	123.16±13.16 ^{###,^^^}

^{***}p<0.001 reveals notably variation of group 3 vs group 4; ^{###}p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; [^]p<0.05, ^{^^}p<0.01, ^{^^^}p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way ANALYSIS OF VARIANCE come succed by Tukey's comparsion test

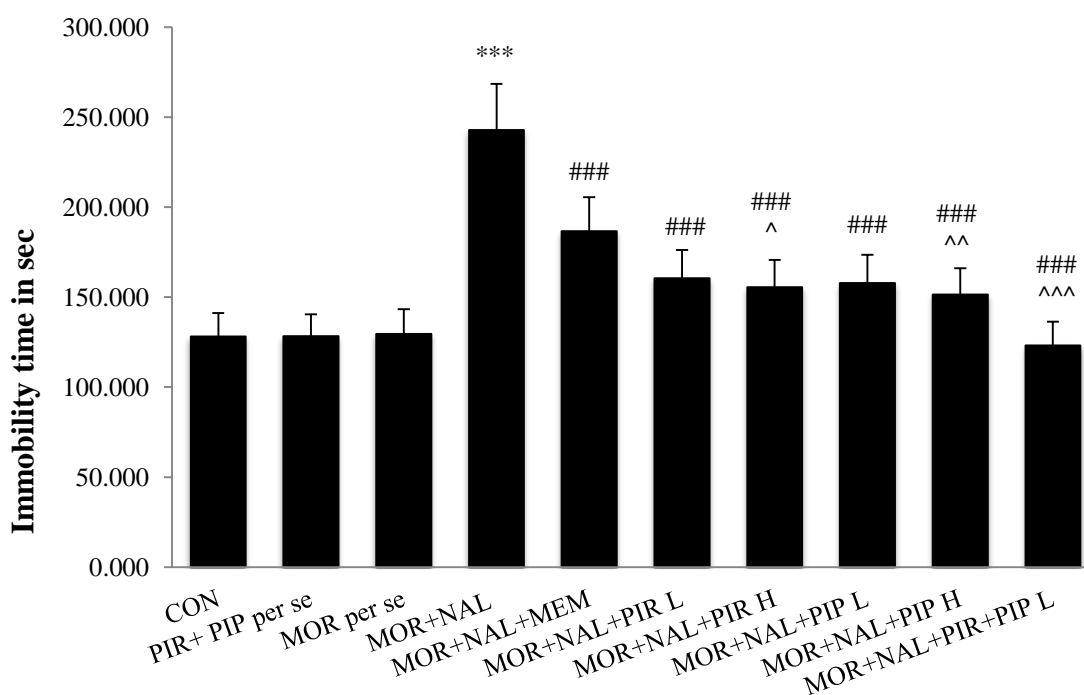


Fig. 12: Effect of pirfenidone and piperine on Depressant activity

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. ^{***}p<0.001 represent significant difference in group 3 vs group 4; ^{###}p<0.001

represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; $\hat{p}<0.05$, $\hat{\hat{p}}<0.01$, $\hat{\hat{\hat{p}}}<0.001$ represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.1.8. Effect of pirfenidone and piperine on anxiety no. of entries in the open arm activity

Administration of morphine *per se* to morphine *per se* group and pirfenidone + piperine to pirfenidone-piperine *per se* group mice for 5 consecutive days, increased the no. of entries in open arm of elevated plus maze apparatus means they did not showed any significant change in anxiety behavior, as compared to vehicle treated control group. But when administration of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days followed by a single injection of naloxone (8 mg/kg; i.p.) 2 hours after morphine injection to group 4 mice decreased the no of entries in open arm, as it showed that animals feel more anxiety as reflected by a significant ($p<0.001$) increase in anxiety behavioral of mice, when compared to morphine *per se* treated group.

The anxiety behavior seen due to precipitation of naloxone-induced morphine withdrawal symptoms were significantly ($p<0.001$) and dose dependently increased in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine-naloxone treated group. The prime effect was observed in pirfenidone (200 and 300 mg/kg; p.o.) ($p<0.01$), piperine (15 mg/kg) ($p<0.01$) and combination treated groups which was significantly ($p<0.001$) better than the standard memantine treated group. Thus, this combination treatment attenuated the morphine withdrawal symptoms as depicted by anxiety behavior in morphine dependent mice.

Table 11: Effect of pirfenidone and piperine on anxiety no. of entries in the open arm activity

Group no.	Group name	No. of entries in open arm Mean \pm SD
1.	Control	8.83 \pm 0.98
2.	Pirfenidone + Piperine	9.00 \pm 0.89
3.	Morphine <i>per se</i>	8.33 \pm 0.82
4.	Morphine + Naloxone	3.67 \pm 0.52 ^{***}
5.	Morphine + Naloxone+ Memantine	5.50 \pm 1.05 ^{###}
6.	Morphine + Naloxone+ Pirfenidone low dose	7.33 \pm 0.82 ^{###, ^^}
7.	Morphine + Naloxone+ Pirfenidone high dose	6.83 \pm 0.75 ^{###, ^^}
8.	Morphine + Naloxone+ Piperine low dose	6.67 \pm 0.52 ^{###}
9.	Morphine + Naloxone+ Piperine high dose	7.67 \pm 0.82 ^{###, ^^}
10.	Morphine + Naloxone+ Pirfenidone + Piperine low dose	8.17 \pm 0.75 ^{###, ^^}

^{***}p<0.001 reveals notably variation group 3 vs group 4; ^{###}p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^{^^}p<0.01, ^{^^^}p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way ANALYSIS OF VARIANCE come succed by Tukey's comparsion test.

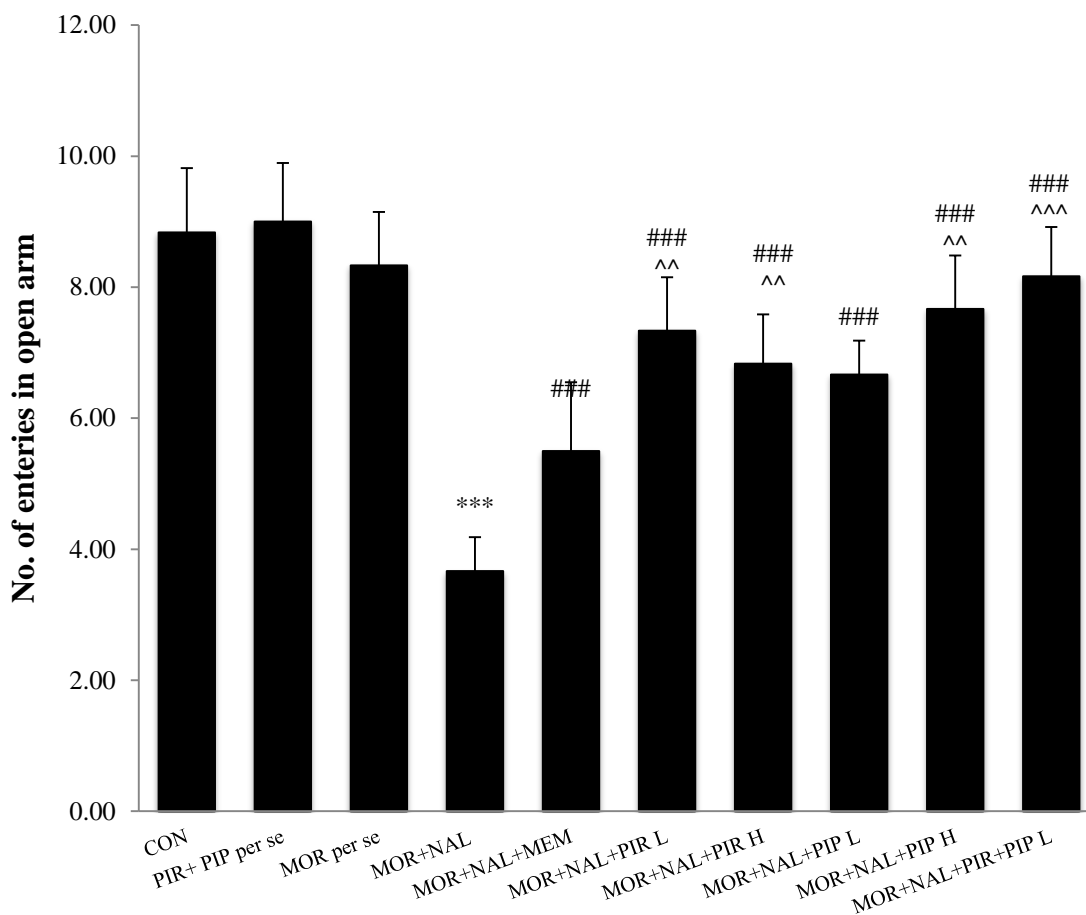


Fig.13: Effect of pirfenidone and piperine on anxiety no. of entries in the open arm activity

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. *** $p < 0.001$ represent significant difference in group 3 vs group 4; ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^ $p < 0.01$, ^^ $p < 0.001$ represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.1.9. Effect of pirfenidone and piperine on anxiety time spent in the open arm activity

It was observed that when administration of morphine *per se* to morphine *per se* group and pirfenidone + piperine to pirfenidone-piperine *per se* group mice for 5 consecutive days, animals spent more time in open arm of elevated plus maze apparatus means they did not showed any significant change in anxiety behavior, as

compared to vehicle treated control group. But when administration of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days followed by a single injection of naloxone (8 mg/kg; i.p.) 2 hours after morphine injection to morphine naloxone group mice spent less time in open arm and more time in closed arm, as it showed that animals feel more anxiety as reflected by a significant ($p < 0.001$) increase in anxiety behavioral of mice, when compared to morphine *per se* treated group.

The anxiety behavior seen due to precipitation of naloxone-induced morphine withdrawal symptoms were significantly and dose dependently reduced in the memantine (10 mg/kg; p.o.) ($p < 0.01$), pirfenidone (200 mg/kg; p.o.) ($p < 0.05$) and (300 mg/kg; p.o.) ($p < 0.01$), piperine (10 and 15 mg/kg; p.o.) ($p < 0.001$) and combination (pirfenidone - 200 mg/kg + piperine- 10 mg/kg; p.o.) ($p < 0.001$) treated groups, when compared to morphine-naloxone treated group. The supreme effect was observed in piperine (15 mg/kg) ($p < 0.01$) and combination treated groups which was significantly ($p < 0.01$) better than the standard memantine treated group. Thus, this combination treatment attenuated the morphine withdrawal symptoms as depicted by anxiety behavior in morphine dependent mice.

Table 12: Effect of pirfenidone and piperine on anxiety time spent in the open arm activity

Group no.	Group name	Time spent in open arm Mean \pm SD
1.	Control	123.50 \pm 12.78
2.	Pirfenidone + Piperine	118.50 \pm 12.12
3.	Morphine <i>per se</i>	125.33 \pm 13.79
4.	Morphine + Naloxone	69.83 \pm 5.19 ^{***}
5.	Morphine + Naloxone+ Memantine	95.83 \pm 10.26 ^{##}
6.	Morphine + Naloxone+ Pirfenidone low dose	95.16 \pm 7.85 [#]
7.	Morphine + Naloxone+ Pirfenidone high dose	107.50 \pm 11.97 ^{##}

8.	Morphine + Naloxone+ Piperine low dose	89.83±12.10 ^{###}
9.	Morphine + Naloxone+ Piperine high dose	104.00±4.42 ^{###, ^^}
10.	Morphine + Naloxone+ Pirfenidone + Piperine low dose	120.00±5.76 ^{###, ^^}

***p<0.001 reveals notably variation of group 3 vs group 4; #p<0.05, ##p<0.01, ###p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^^p<0.01, represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way ANALYSIS OF VARIANCE come succed by Tukey's comparsion test

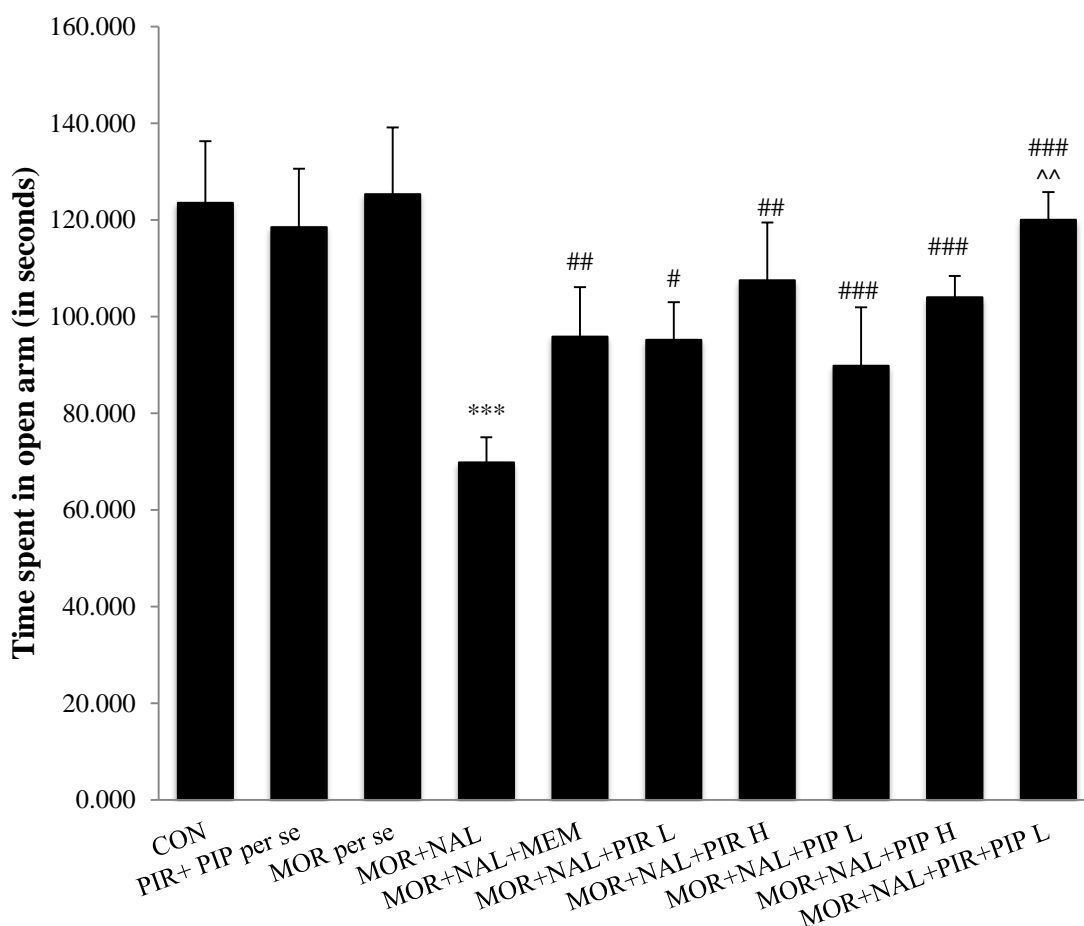


Fig. 14: Effect of pirfenidone and piperine on anxiety time spent in open arm activity

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine ; NAL: Naloxone; MEM: Memantine; L: low dose; H: high

dose, *** $p < 0.001$ represent significant difference in group 3 vs group 4; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^ $p < 0.05$, ^^ $p < 0.01$, represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.1.10. Effect of pirfenidone and piperine on analgesic MPE % activity

It was observed that when administration of morphine *per se* to morphine *per se* group and pirfenidone + piperine to pirfenidone piperine *per se* group mice for 5 consecutive days, animals spent more time in open arm of elevated plus maze apparatus means they did not showed any significant change in anxiety behavior, as compared to vehicle treated control group. But when administration of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days followed by a single injection of naloxone (8 mg/kg; i.p.) 2 hours after morphine injection to group 4 mice spent less time in open arm and more time in closed arm, as it showed that animals feel more anxiety as reflected by a significant ($p < 0.001$) increase in anxiety behavioral of mice, when compared to morphine *per se* treated group.

The anxiety behavior seen due to precipitation of naloxone-induced morphine withdrawal symptoms were significantly and dose dependently reduced in the memantine (10 mg/kg; p.o.) ($p < 0.01$), pirfenidone (200 mg/kg; p.o.) ($p < 0.05$) and (300 mg/kg; p.o.) ($p < 0.01$), piperine (10 and 15 mg/kg; p.o.) ($p < 0.001$) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) ($p < 0.001$) treated groups, when compared to morphine-naloxone treated group. The supreme effect was observed in piperine (15 mg/kg) ($p < 0.01$) and combination treated groups which was significantly ($p < 0.01$) better than the standard memantine treated group. Thus, this combination treatment attenuated the morphine withdrawal symptoms as depicted by anxiety behavior in morphine dependent mice.

Table 13: Effect of pirfenidone and piperine on analgesic MPE % activity

Group no.	Group name	MPE% Mean \pm SD
1.	Control	6.33 \pm 1.03
2.	Pirfenidone + Piperine	6.33 \pm 5.49 ^{\$\$\$}
3.	Morphine <i>per se</i>	63.50 \pm 6.72 ^{\$\$\$}
4.	Morphine + Naloxone	32.33 \pm 3.20 ^{***}
5.	Morphine + Naloxone+ Memantine	43.50 \pm 4.81 ^{##}
6.	Morphine + Naloxone+ Pirfenidone low dose	37.83 \pm 4.12
7.	Morphine + Naloxone+ Pirfenidone high dose	46.00 \pm 6.69 ^{###}
8.	Morphine + Naloxone+ Piperine low dose	41.83 \pm 4.49 ^{###}
9.	Morphine + Naloxone+ Piperine high dose	48.17 \pm 4.54 ^{###}
10.	Morphine + Naloxone+ Pirfenidone + Piperine low dose	58.17 \pm 6.15 ^{###, ^^}

^{\$\$\$}p<0.001 represent significant difference in group 1 vs. group 2 and 3; ^{***}p<0.001 represent significant difference in group 3 vs group 4; ^{##}p<0.01, ^{###}p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^{^^}p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way analysis of variance come succeed by Tukey's comparison test.

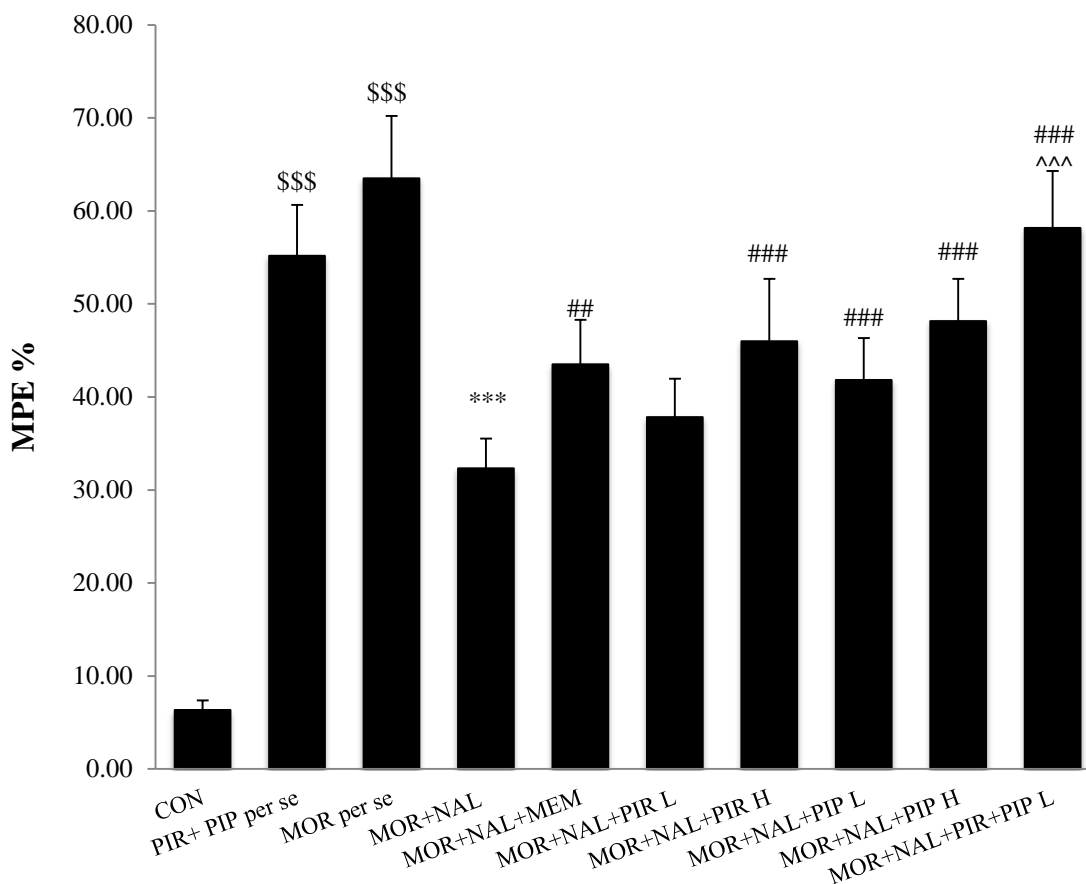


Fig. 15: Effect of pirfenidone and piperine on analgesic MPE % activity

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. \$\$\$ $p < 0.001$ represent significant difference in group 1 vs. group 2 and 3; *** $p < 0.001$ represent significant difference in group 3 vs group 4; ## $p < 0.01$, ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^^ $p < 0.001$ represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.1.11. Effect of pirfenidone and piperine on Defecation frequency

It was observed that, no significant change in defecation of mice frequency when administration of morphine *per se* to morphine *per se* group and pirfenidone + piperine to pirfenidone-piperine *per se* group mice for 5 consecutive days as compared to vehicle treated control group. When administration of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days followed by a single injection of

naloxone (8 mg/kg; i.p.) 2 hours after morphine injection to group 4 mice precipitated the withdrawal syndrome, as reflected by a significant ($p < 0.001$) increase the frequency of defecation in mice, when compared to morphine *per se* treated group.

Increased in defecation frequency had been seen due to precipitation of naloxone-induced morphine withdrawal symptoms were significantly ($p < 0.001$) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone-200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine-naloxone treated group. The foremost effect was observed in combination treated groups which was significantly ($p < 0.01$) better than the standard memantine treated group. Thus, this combination treatment attenuated the morphine withdrawal symptoms as depicted by reduced defecation frequency in morphine dependent mice.

Table 14: Effect of pirfenidone and piperine on Defecation frequency

Group no.	Group name	Defecation frequency Mean \pm SD
1.	Control	1.33 \pm 0.17
2.	Pirfenidone + Piperine	1.50 \pm 0.17
3.	Morphine <i>per se</i>	1.16 \pm 0.14
4.	Morphine + Naloxone	4.33 \pm 0.17 ^{***}
5.	Morphine + Naloxone+ Memantine	2.66 \pm 0.18 ^{###}
6.	Morphine + Naloxone+ Pirfenidone low dose	3.00 \pm 0.15 ^{###}
7.	Morphine + Naloxone+ Pirfenidone high dose	1.66 \pm 0.17 ^{###}
8.	Morphine + Naloxone+ Piperine low dose	2.00 \pm 10.16 ^{###}
9.	Morphine + Naloxone+ Piperine high dose	1.66 \pm 0.15 ^{###}
10.	Morphine + Naloxone+ Pirfenidone + Piperine low	1.00 \pm 0.13 ^{###, ^}

	dose	
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*** $p < 0.001$ represent significant difference in group 3 vs group 4; ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^ $p < 0.01$, represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way analysis of variance followed by Tukey's comparison test

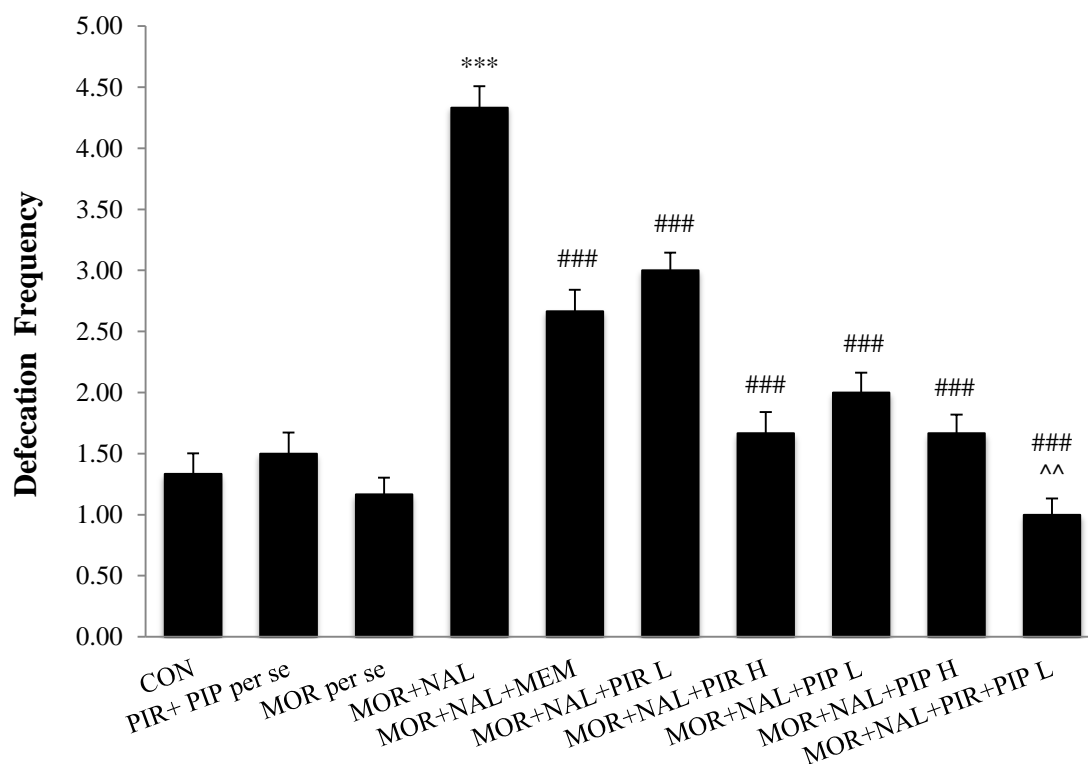


Fig. 16: Effect of pirfenidone and piperine on Defecation frequency

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. *** $p < 0.001$ represent significant difference in group 3 vs group 4; ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^ $p < 0.01$, represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.1.12. Effect of pirfenidone and piperine on urination frequency

It has been seen that, administration of morphine *per se* to morphine *per se* group and pirfenidone + piperine to pirfenidone-piperine *per se* group 2 mice mice

for 5 consecutive days did not showed any significant change in mice urination frequency, as compared to vehicle treated control group. But when administration of morphine (5mg/kg; i.p.) twice daily for a period of 5 days followed by a single injection of naloxone (8mg/kg; i.p.) 2 hours after morphine injection to group 4 mice precipitated the withdrawal syndrome, as reflected by a significant ($p < 0.001$) increase in urination of mice, when compared to morphine *per se* treated group.

Increased in urination seen due to precipitation of naloxone-induced morphine withdrawal symptoms were significantly ($p < 0.001$) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine-naloxone treated group. The best effect was observed in combination treated groups which was significantly ($p < 0.001$) better than the standard memantine treated group, pirfenidone (200 and 300 mg/kg) and piperine (10 and 15 mg/kg). Thus, the combination of both i.e pirfenidone and piperine (200 and 10 mg/kg respectively). Treatment attenuated the morphine withdrawal symptoms as depicted by reduced urine frequency in morphine dependent mice.

Table 15: Effect of pirfenidone and piperine on urination frequency

Group no.	Group name	Urine frequency Mean \pm SD
1.	Control	0.33 \pm 0.52
2.	Pirfenidone + Piperine	0.16 \pm 0.41
3.	Morphine <i>per se</i>	0.16 \pm 0.41
4.	Morphine + Naloxone	7.16 \pm 0.75***
5.	Morphine + Naloxone+ Memantine	4.16 \pm 0.41###
6.	Morphine + Naloxone+ Pirfenidone low dose	3.66 \pm 0.52###
7.	Morphine + Naloxone+ Pirfenidone high dose	3.50 \pm 0.55###
8.	Morphine + Naloxone+ Piperine low dose	3.33 \pm 0.52###

9.	Morphine + Naloxone+ Piperine high dose	3.50±0.84 ^{###}
10.	Morphine + Naloxone+ Pirfenidone + Piperine low dose	1.50±0.548 ^{###, ^^}

^{***}p<0.001 reveals notably variation of group 3 vs group 4; ^{###}p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^{^^}p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way ANALYSIS OF VARIANCE come succeed by Tukey's comparison test

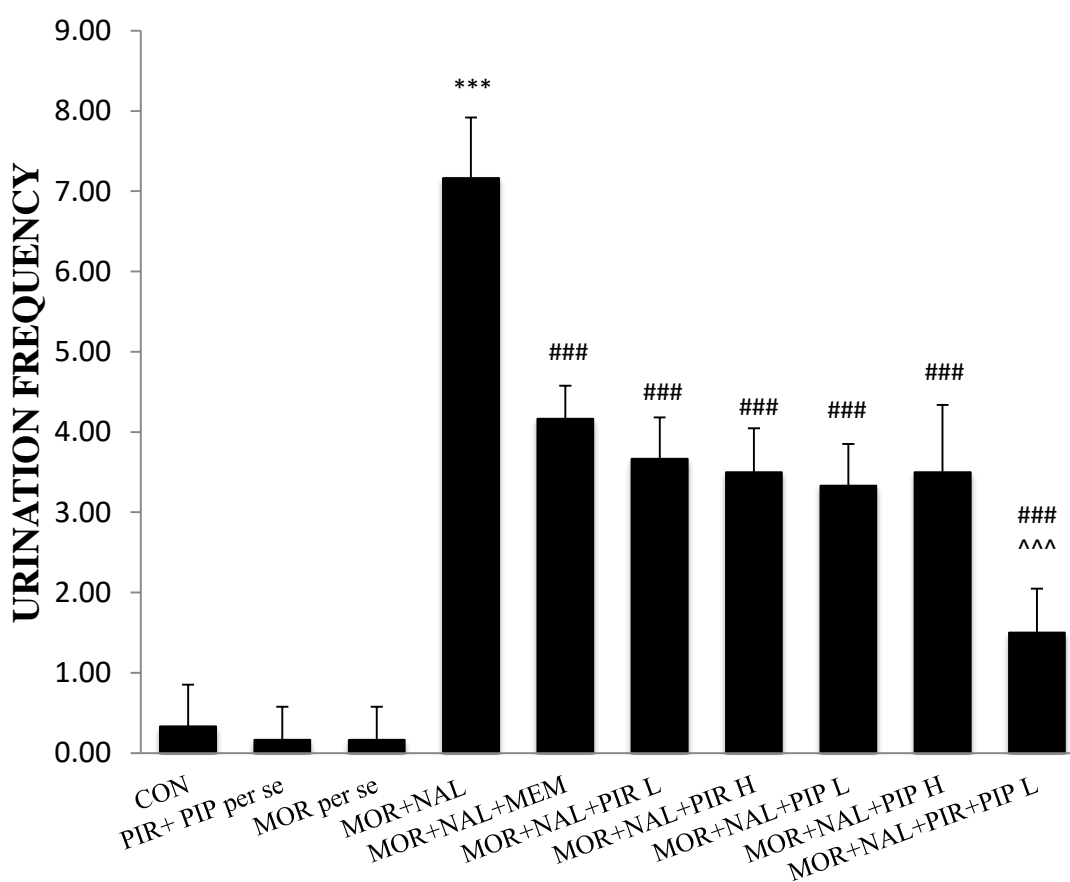


Fig. 17: Effect of pirfenidone and piperine on urination frequency

Data represented as mean ± SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose

^{***}p<0.001 represent significant difference in group 3 vs group 4; ^{###}p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^{^^}p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.2. Behavioral evaluations of the spontaneous animal model.

5.1.2.1. Effect of pirfenidone and piperine on jumping frequency

Administration of pirfenidone + piperine to pirfenidone-piperine *per se* group and morphine *per se* to morphine *per se* group in mice for 5 consecutive days did not showed any significant change in mice jumping frequency, as compared to vehicle treated control group. Administration of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days to morphine naloxone group in mice and discontinue the drug on 6th day, increase in the withdrawal syndrome, as reflected by a significant ($p < 0.001$) increase in jumping behavioral of mice, when compared to morphine *per se* treated group.

The stereotyped jumping behavior seen due to termination of morphine and seen the spontaneous withdrawal symptoms which were significantly ($p < 0.001$) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone-200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine withdrawal group. The best effect was observed combination treated groups which was significantly ($p < 0.001$) better than the standard memantine treated group, pirfenidone (200 and 300 mg/kg) and piperine (10 and 15 mg/kg). Thus, this type of combination treatment attenuated the morphine withdrawal symptoms as depicted by reduced jumping behavior in morphine dependent mice.

Table 16: Effect of pirfenidone and piperine on jumping frequency

Group no.	Group name	Jumping frequency Mean \pm SD
1.	Control	5.00 \pm 0.33
2.	Pirfenidone + Piperine	4.66 \pm 0.32
3.	Morphine <i>per se</i>	4.83 \pm 0.34
4.	Morphine Withdrawal	11.17 \pm 0.31 ^{***}

5.	Morphine + Memantine	8.17±0.36 ^{###}
6.	Morphine + Pirfenidone low dose	7.67±0.32 ^{###}
7.	Morphine + Pirfenidone high dose	6.67±0.31 ^{###}
8.	Morphine + Piperine low dose	7.33±0.33 ^{###}
9.	Morphine + Piperine high dose	7.17±0.57 ^{###}
10.	Morphine + Pirfenidone + Piperine low dose	5.50±0.55 ^{###, ^}

*** p<0.001 reveals notably variation of group 3 vs group 4; ### p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^ p<0.01, represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way analysis of variance come succeed by Tukey's comparison test

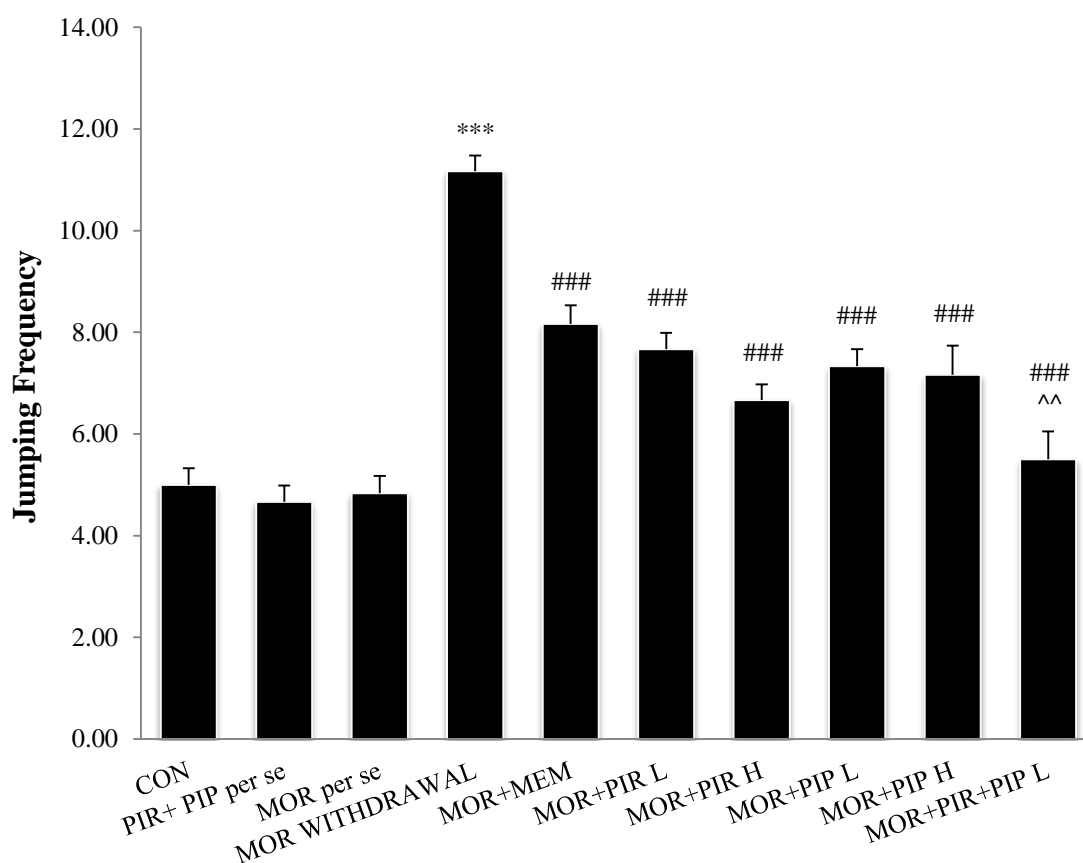


Fig. 18: Effect of pirfenidone and piperine on jumping frequency

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. *** $p < 0.001$ represent significant difference in group 3 vs group 4 ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^ $p < 0.01$, represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.2.2. Effect of pirfenidone and piperine on circling frequency

It was observed that administration of pirfenidone + piperine to pirfenidone-piperine *per se* group and morphine *per se* to morphine *per se* group in mice for 5 consecutive days did not showed any significant change in mice circling frequency, as compared to vehicle treated control group. Administration of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days to morphine naloxone group in mice and discontinue the drug on 6th day, increase in the withdrawal syndrome, as reflected by a significant ($p < 0.001$) increase in circling behavioral of mice, when compared to morphine *per se* treated group.

The stereotyped circling behavior seen due to termination of morphine and seen the spontaneous withdrawal symptoms which were significantly ($p < 0.001$) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine withdrawal group. The best effect was observed Pirfenidone (200 mg/kg; p.o) ($p < 0.05$) combination treated groups (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) which was significantly ($p < 0.001$) better than the standard memantine treated group, pirfenidone (200 and 300 mg/kg) and piperine (10 and 15 mg/kg). Thus, this type of combination treatment attenuated the morphine withdrawal symptoms as depicted by reduced circling behavior in morphine dependent mice.

Table 17: Effect of pirfenidone and piperine on circling frequency

Group no.	Group name	Circling frequency Mean \pm SD
1.	Control	5.17 \pm 1.17
2.	Pirfenidone + Piperine	4.67 \pm 0.82
3.	Morphine <i>per se</i>	4.67 \pm 0.52
4.	Morphine Withdrawal	18.17 \pm 1.94 ^{***}
5.	Morphine + Memantine	15.17 \pm 1.47 ^{###}
6.	Morphine + Pirfenidone low dose	13.00 \pm 2.10 ^{###^}
7.	Morphine + Pirfenidone high dose	12.00 \pm 1.55 ^{###}
8.	Morphine + Piperine low dose	13.33 \pm 2.50 ^{###}
9.	Morphine + Piperine high dose	12.17 \pm 1.17 ^{###}
10.	Morphine + Pirfenidone + Piperine low dose	7.83 \pm 1.47 ^{##, ^^}

^{***} p<0.001 represent significant difference ingroup 3 vs group 4; ^{###} p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; [^] p<0.05, ^{^^} p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way ANALYSIS OF VARIANCE come succeed by Tukey's comparsion test.

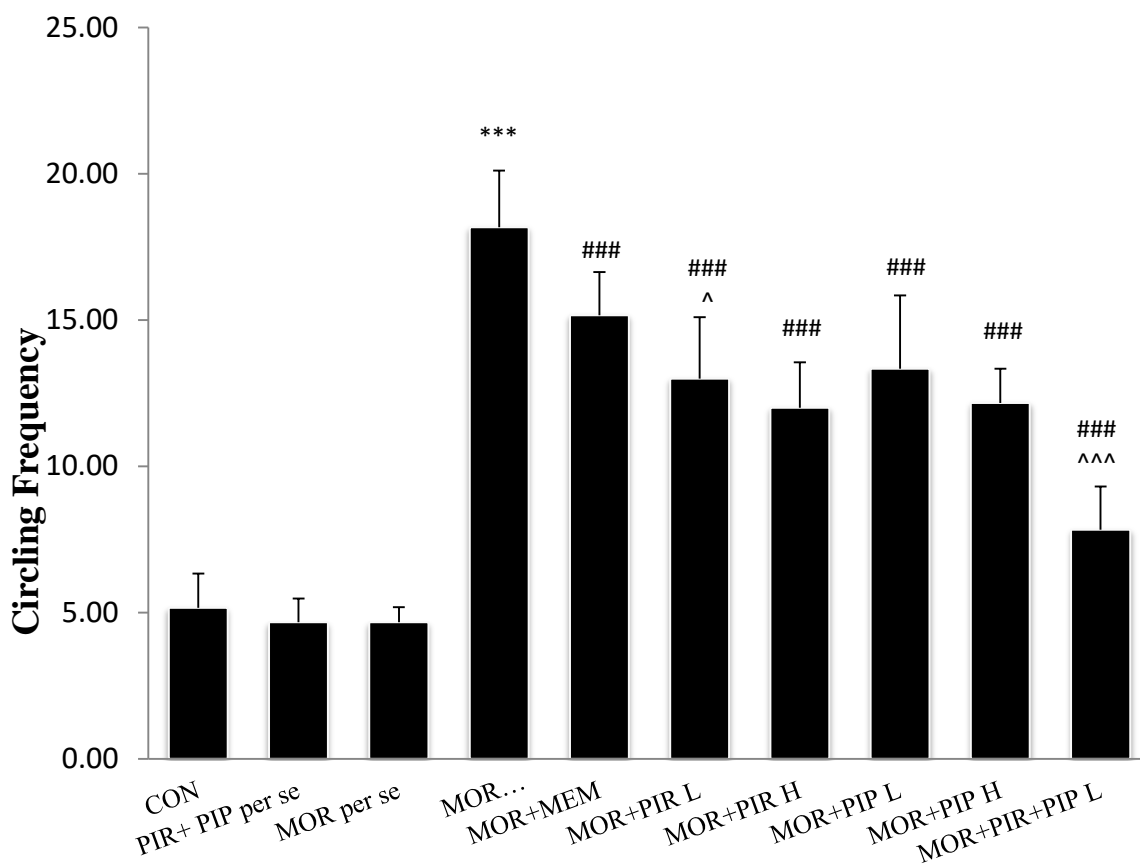


Fig. 18: Effect of pirfenidone and piperine on circling frequency

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. *** $p < 0.001$ represent significant difference in group 3 vs group 4; ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^ $p < 0.05$, ^^ $p < 0.001$ represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.2.3. Effect of pirfenidone and piperine on rearing frequency

It was seen that administration of pirfenidone + piperine to pirfenidone-piperine *per se* group and morphine *per se* to morphine *per se* group in mice for 5 consecutive days did not show any significant change in mice rearing frequency, as compared to vehicle treated control group. Administration of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days to morphine naloxone group in mice and discontinuation of the drug on 6th day, increase in the withdrawal syndrome, as reflected by

a significant ($p<0.001$) increase in rearing behavioral of mice, when compared to morphine *per se* treated group.

The rearing behavior seen due to termination of morphine and seen the spontaneous withdrawal symptoms which were significantly ($p<0.01$) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirfenidone ($p<0.01$) (300 mg/kg; p.o.), piperine ($p<0.01$) (15 mg/kg; p.o.) and combination ($p<0.001$) (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine withdrawal group. The best effect was observed in combination treated groups (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) which was significantly ($p<0.01$) better than the standard memantine treated group, pirfenidone (200 and 300 mg/kg) and piperine (10 and 15 mg/kg). Thus, this type of combination treatment attenuated the morphine withdrawal symptoms as depicted by reduced rearing behavior in morphine dependent mice.

Table 18: Effect of pirfenidone and piperine on Rearing frequency

Group no.	Group name	Rearing frequency Mean \pm SD
1.	Control	4.50 \pm 0.34
2.	Pirfenidone + Piperine	4.83 \pm 0.31
3.	Morphine <i>per se</i>	5.00 \pm 0.32
4.	Morphine Withdrawal	7.33 \pm 0.33 ^{***}
5.	Morphine + Memantine	6.00 \pm 0.35 ^{###}
6.	Morphine + Pirfenidone low dose	5.67 \pm 0.35 ^{###, ^^}
7.	Morphine + Pirfenidone high dose	5.22 \pm 0.31 ^{###, ^^}
8.	Morphine + Piperine low dose	5.12 \pm 0.39 ^{###, ^^}
9.	Morphine + Piperine high dose	5.22 \pm 0.34 ^{###, ^}
10.	Morphine + Pirfenidone + Piperine low dose	4.00 \pm 0.35 ^{###, ^^}

*** $p < 0.001$ represent significant difference in group 3 vs group 4; ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^ $p < 0.05$, ^^ $p < 0.01$, ^^ $p < 0.001$ represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way ANALYSIS OF VARIANCE come succed by Tukey's comparson test

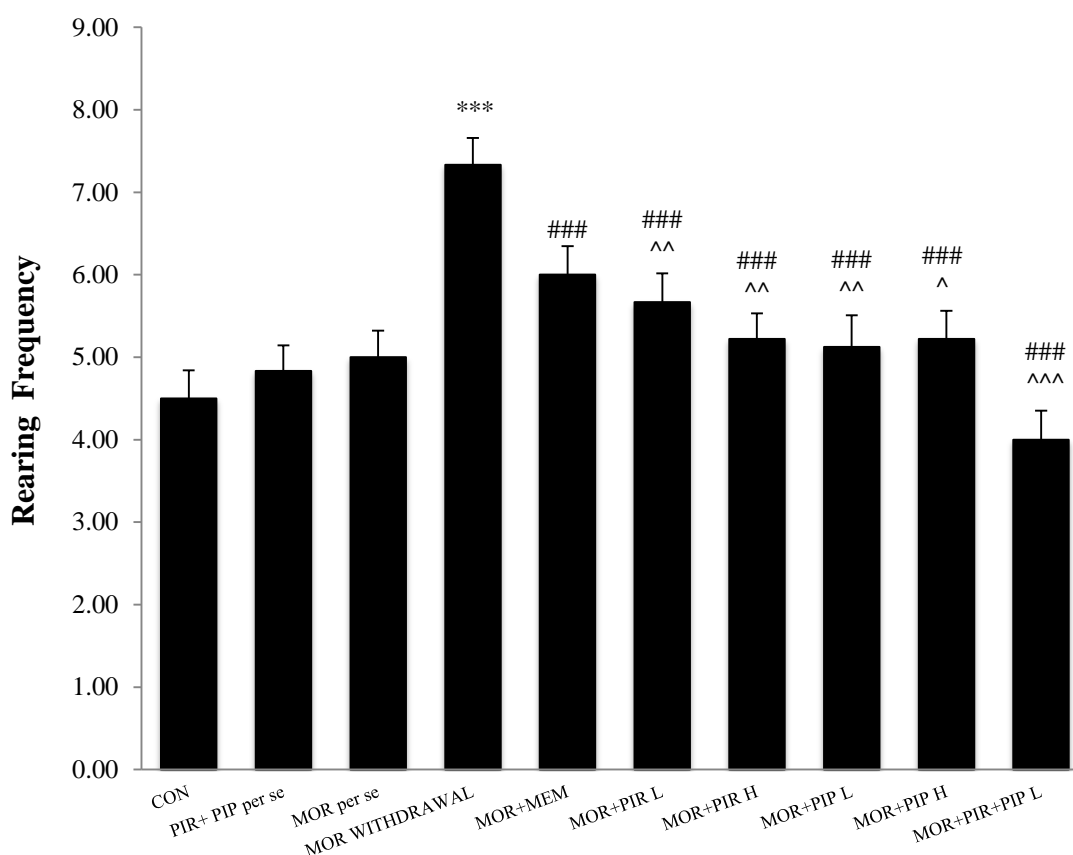


Fig. 20: Effect of pirfenidone and piperine on rearing frequency.

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose.

*** $p < 0.001$ represent significant difference in group 3 vs group 4; ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^ $p < 0.05$, ^^ $p < 0.01$, ^^ $p < 0.001$ represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.2.4. Effect of pirfenidone and piperine on forepaw licking frequency

It was seen that administration of pirfenidone + piperine to pirfenidone-piperine *per se* group and morphine *per se* to morphine *per se* group in mice for 5

consecutive days did not showed any significant change in mice rearing frequency, as compared to vehicle treated control group. Administration of morphine (5mg/kg; i.p.) twice daily for a period of 5 days to morphine naloxone group in mice and discontinue the drug on 6th day, increase in the withdrawal syndrome, as reflected by a significant ($p<0.001$) increase in rearing behavioral of mice, when compared to morphine *per se* treated group.

The rearing behavior seen due to termination of morphine and seen the spontaneous withdrawal symptoms which were significantly ($p<0.01$) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pifenidone ($p<0.01$) (300 mg/kg; p.o.), piperine ($p<0.01$) (15 mg/kg; p.o.) and combination ($p<0.001$) (pifenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine withdrawal group. The best effect was observed in combination treated groups (pifenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) which was significantly ($p<0.01$) better than the standard memantine treated group, pifenidone (200 and 300 mg/kg) and piperine (10 and 15 mg/kg). Thus, this type of combination treatment attenuated the morphine withdrawal symptoms as depicted by reduced rearing behavior in morphine dependent mice.

Table 19: Effect of pifenidone and piperine on forepaw licking frequency

Group no.	Group name	Fore paw licking frequency Mean \pm SD
1.	Control	2.83 \pm 0.40
2.	Pirfenidone + Piperine	2.67 \pm 0.51
3.	Morphine <i>per se</i>	2.83 \pm 1.47
4.	Morphine Withdrawal	4.50 \pm 0.83 ^{***}
5.	Morphine + Memantine	3.00 \pm 0.89 ^{###}
6.	Morphine + Pirfenidone low dose	2.67 \pm 1.21 ^{###}
7.	Morphine + Pirfenidone high dose	2.50 \pm 1.37 ^{###,^}

8.	Morphine + Piperine low dose	2.83±0.75 ^{###}
9.	Morphine + Piperine high dose	3.00±0.89 ^{###}
10.	Morphine + Pirfenidone + Piperine low dose	2.17±0.40 ^{###, ^^}

^{***}p<0.001 represent significant difference in group 3 vs group 4; ^{###}p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; [^]p<0.05, ^{^^}p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way ANALYSIS OF VARIANCE come succeed by Tukey's comparison test.

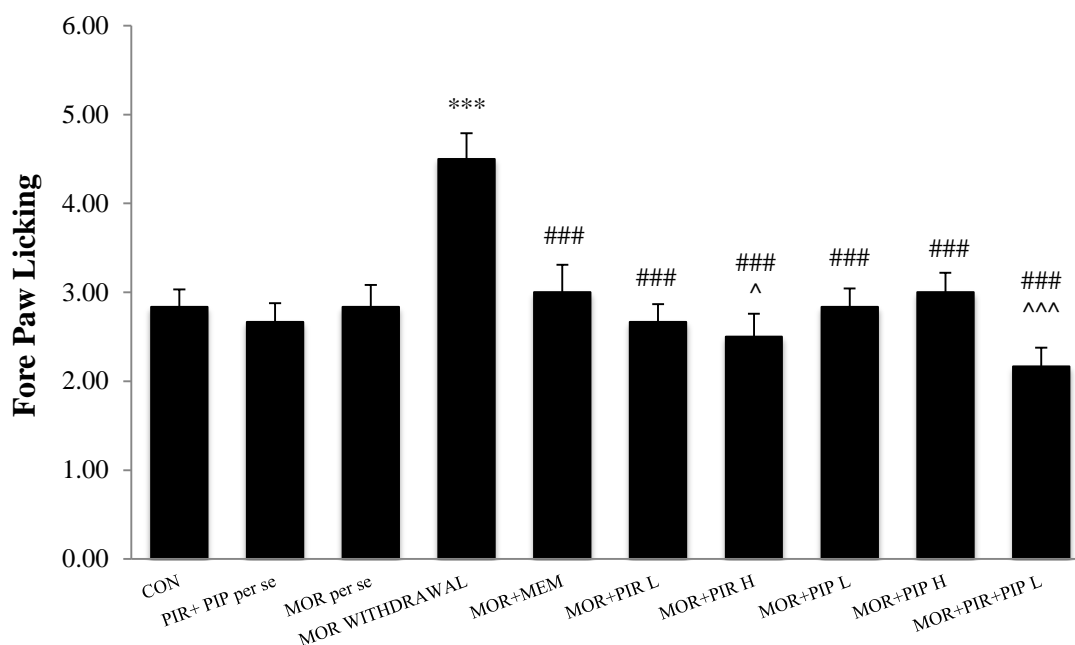


Fig. 21: Effect of pirfenidone and piperine on fore paw licking frequency

Data represented as mean \pm standard deviation were statistically examined using one-way Analysis of variance come succeed by Tukey's comparison test. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; MEM: Memantine; L: low dose; H: high dose. ^{\$}p<0.05, ^{\$\$}p<0.01, ^{\$\$\$}p<0.001 represent significant difference in group 1 vs. group 2 and 3; ^{*}p<0.05, ^{**}p<0.01, ^{***}p<0.001 represent significant difference in group 3 vs group 4; [#]p<0.05, ^{##}p<0.01, ^{###}p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; [^]p<0.05, ^{^^}p<0.01, ^{^^^}p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.2.5. Effect of pirfenidone and piperine on withdrawal chronic score WSS

Administration of pirfenidone + piperine to pirfenidone-piperine *per se* group and morphine *per se* to morphine *per se* group in mice for 5 consecutive days did not showed any significant change in mice on withdrawal severity score, as compared to vehicle treated control group. But when morphine was administered (5mg/kg; i.p.) twice daily for a period of 5 days to morphine naloxone *per se* group in mice and discontinue the drug on 6th day, increase in the withdrawal syndrome, as reflected by a significant ($p < 0.001$) increase in fore paw licking behavioral of mice, when compared to morphine *per se* treated group.

The withdrawal severity score behavior seen due to termination of morphine and seen the spontaneous withdrawal symptoms which were significantly and dose dependently reduced in the memantine ($p < 0.05$) (10 mg/kg; p.o.), pirfenidone ($p < 0.01$) (200 mg/kg; p.o.), pirfenidone ($P < 0.001$) (300 mg/kg; p.o.), piperine ($P < 0.001$) (10 and 15 mg/kg; p.o.) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine withdrawal group. The best effect was observed in pirfenidone ($p < 0.001$) (300 mg/kg; p.o), piperine ($p < 0.001$) (15 mg/kg; p.o) and combination treated groups (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) which was significantly better than the standard memantine treated group, pirfenidone (200 mg/kg) and piperine (10 mg/kg). Thus, this type of combination treatment (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.), pirfenidone (300 mg/kg; p.o) and piperine (15 mg/kg; p.o) attenuated the morphine withdrawal symptoms as depicted by reduced withdrawal severity score behavior in morphine dependent mice.

Table 20: Effect of pirfenidone and piperine on withdrawal chronic score WSS

Group no.	Group name	WSS frequency Mean \pm SD
1.	Control	2.17 \pm 0.23
2.	Pirfenidone + Piperine	2.33 \pm 0.23

3.	Morphine <i>per se</i>	2.00±0.25
4.	Morphine Withdrawal	10.00±0.30 ^{***}
5.	Morphine + Memantine	8.50±0.36 [#]
6.	Morphine + Pirfenidone low dose	8.33±0.33 ^{##}
7.	Morphine + Pirfenidone high dose	5.83±0.43 ^{###, ^^}
8.	Morphine + Piperine low dose	7.00±0.36 ^{###, ^}
9.	Morphine + Piperine high dose	6.00±0.35 ^{###, ^^}
10.	Morphine + Pirfenidone + Piperine low dose	3.83±0.37 ^{###, ^^}

^{***}p<0.001 represent significant difference in group 3 vs group 4; #p<0.05, ##p<0.01, ###p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^p<0.05, ^^p<0.01, ^^p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way analysis of variance come succeed by Tukey's comparison test.

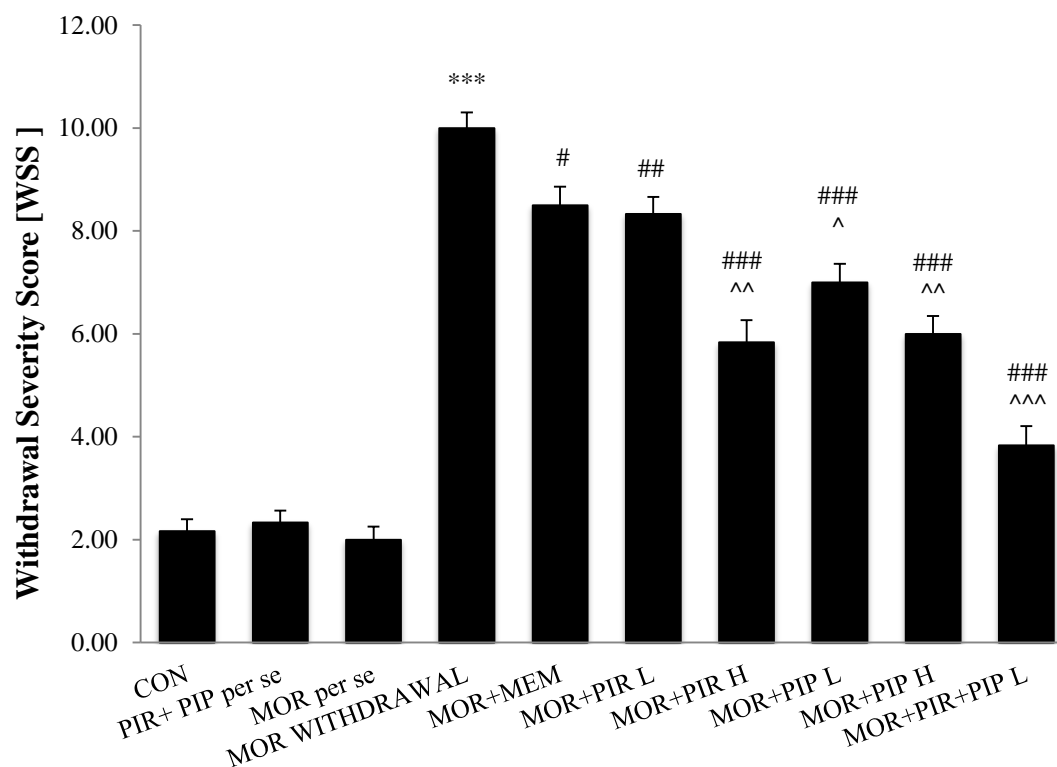


Fig. 22: Effect of pirfenidone and piperine on withdrawal chronic score

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. *** $p < 0.001$ represent significant difference in group 3 vs group 4; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^ $p < 0.05$, ^^ $p < 0.01$, ^^ $p < 0.001$ represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.2.6. Effect of pirfenidone and piperine on Locomotor activity

There was no change in locomotor activity when administration of morphine *per se* to morphine *per se* group and pirfenidone + piperine to pirfenidone-piperine *per se* group in mice for 5 consecutive days in mice as compared to vehicle treated control group. But when administration of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days to morphine naloxone group 4 mice and discontinue the drug on 6th day, as reflected by a significant ($p < 0.01$) decrease in locomotion behavioral of mice, when compared to morphine *per se* treated group.

The locomotion behavior seen due to discontinuation of morphine on 6th day of the study and seen the spontaneous withdrawal symptoms were significantly and dose dependently increased the counts of locomotion in memantine (10 mg/kg; p.o.) ($p < 0.01$) pirfenidone (200 mg/kg; p.o.) ($p < 0.001$), pirfenidone (300 mg/kg; p.o.) ($p < 0.01$), piperine (10 and 15 mg/kg; p.o.) ($p < 0.001$) and combination (pirfenidone 200 mg/kg + piperine-0 10 mg/kg; p.o.) ($p < 0.001$) treated groups, when compared to morphine withdrawal group. The supreme effect was observed in pirfenidone (300 mg/kg; p.o.) ($p < 0.05$), piperine (15 mg/kg) ($p < 0.01$) and combination treated groups which was significantly ($p < 0.001$) better than the standard memantine treated group, pirfenidone (200 mg/kg) and piperine (10 mg/kg). Thus, this combination treatment attenuated the morphine withdrawal symptoms as depicted by increased the no. of counts in locomotion behavior in morphine dependent mice.

Table 21: Effect of pirfenidone and piperine on Locomotor activity

Group no.	Group name	Counts Mean \pm SD
1.	Control	345.23 \pm 22.20
2.	Pirfenidone + Piperine	317.17 \pm 21.50
3.	Morphine <i>per se</i>	315.33 \pm 22.75
4.	Morphine Withdrawal	251.83 \pm 20.32 ^{**}
5.	Morphine + Memantine	320.25 \pm 12.50 ^{##}
6.	Morphine + Pirfenidone low dose	308.12 \pm 17.58 ^{###}
7.	Morphine + Pirfenidone high dose	314.22 \pm 23.12 ^{##}
8.	Morphine + Piperine low dose	303.10 \pm 21.97 ^{###}
9.	Morphine + Piperine high dose	316.20 \pm 22.12 ^{###}
10.	Morphine + Pirfenidone + Piperine low dose	374.56 \pm 22.98 ^{###, ^^}

^{**}p<0.01 represent significant difference ingroup 3 vs group 4; ^{##}p<0.01, ^{###}p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^{^^}p<0.01 represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way analysis of variance come succeed by Tukey's comparison test.

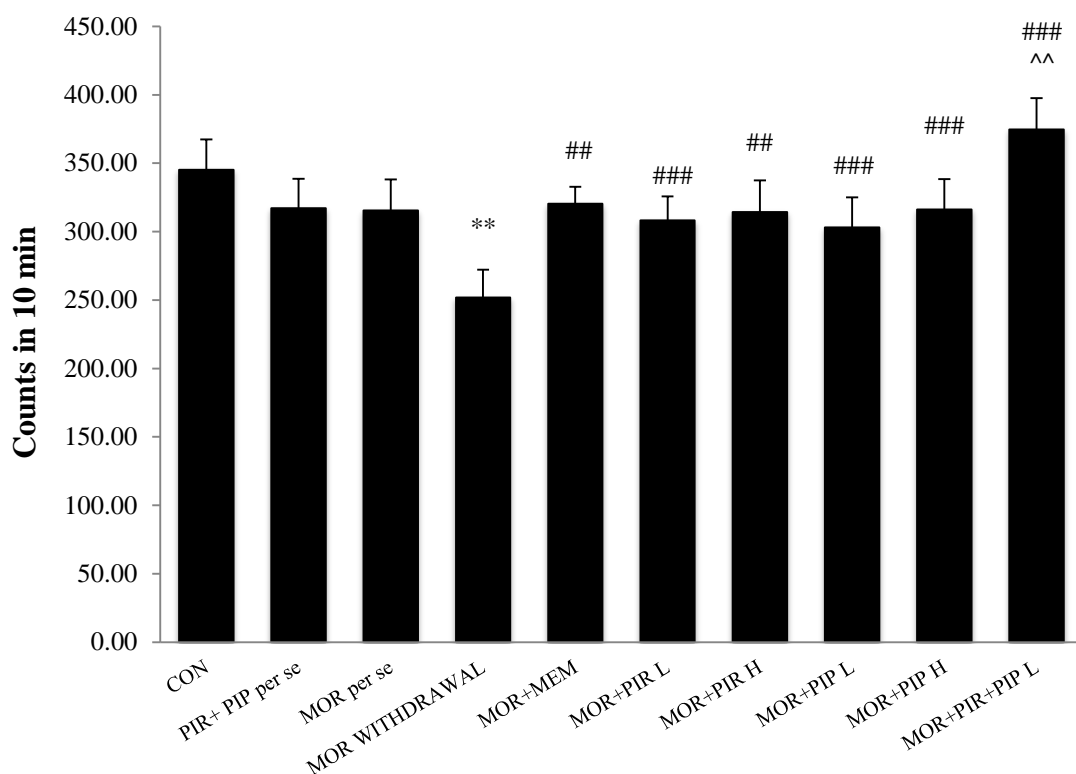


Fig. 23: Effect of pirfenidone and piperine on Locomotor activity

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. ** $p < 0.01$, represent significant difference in group 3 vs group 4; ## $p < 0.01$, ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^^ $p < 0.01$ represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.2.7. Effect of pirfenidone and piperine on depressant activity

One way ANOVA of the total immobility time in mice on day 6 revealed that no significant change in mice when administration of morphine *per se* to morphine *per se* group and pirfenidone + piperine to pirfenidone –piperine *per se* group in mice for 5 consecutive days for depressant behavior as withdrawal syndrome in mice, as compared to vehicle treated control group. But when administration of morphine (5mg/kg; i.p.) twice daily for a period of 5 days and terminate on 6th day to morphine naloxone group in mice showed the spontaneous withdrawal syndrome, as reflected

by a significant ($p < 0.01$) increase in immobility time, when compared to morphine *per se* treated group.

The depressant behavior seen due to disruption of morphine on 6th day causes morphine withdrawal symptoms which were significantly and dose dependently increased in the memantine (10 mg/kg; p.o.) ($p < 0.05$), pirfenidone (200 mg/kg; p.o.) ($p < 0.05$), piperine (15 mg/kg; p.o.) ($p < 0.05$) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine withdrawal group. The good effect was observed in combination treated groups which was significantly ($p < 0.05$) better than the standard memantine treated group, pirfenidone (200 and 300 mg/kg) and piperine (10 and 15 mg/kg). Thus, the combination treatment attenuated the morphine withdrawal symptoms as depicted by reduced the immobility time (depressant behavior) in morphine dependent mice.

Table 22: Effect of pirfenidone and piperine on Depressant activity

Group no.	Group name	Immobility time Mean \pm SD
1.	Control	121.67 \pm 8.10
2.	Pirfenidone + Piperine	122.33 \pm 9.13
3.	Morphine <i>per se</i>	142.50 \pm 10.22
4.	Morphine Withdrawal	180.50 \pm 11.25 ^{***}
5.	Morphine + Memantine	151.00 \pm 10.43 ^{###}
6.	Morphine + Pirfenidone low dose	151.67 \pm 13.12 ^{###}
7.	Morphine + Pirfenidone high dose	155.67 \pm 12.78 ^{###}
8.	Morphine + Piperine low dose	157.83 \pm 12.30 ^{###}
9.	Morphine + Piperine high dose	151.500 \pm 12.10 ^{###}
10.	Morphine + Pirfenidone + Piperine low dose	125.83 \pm 11.10 ^{###, ^^}

^{***} $p < 0.001$ represent significant difference in group 3 vs group 4; ^{###} $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^{^^} $p < 0.01$,

represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way analysis of variance come succeed by Tukey's comparison test

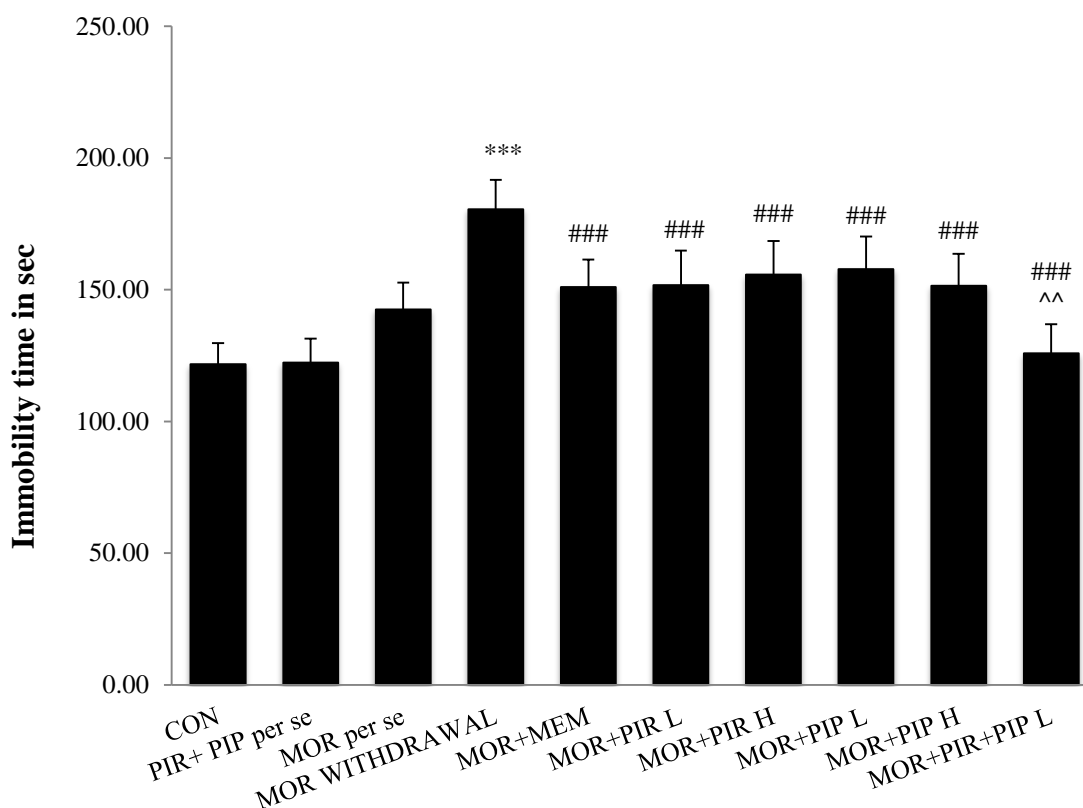


Fig. 24: Effect of pirfenidone and piperine on Depressant activity

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. *** $p < 0.001$ represent significant difference in group 3 vs group 4; ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^^ $p < 0.01$, represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.2.8. Effect of pirfenidone and piperine on anxiety no. of entries in the open arm activity

Administration of morphine *per se* to morphine *per se* group 3 and pirfenidone + piperine to pirfenidone-piperine *per se* group in mice for 5 consecutive days, increased the no. of entries in open arm of elevated plus maze apparatus means they did not showed any significant change in anxiety behavior, as compared to vehicle treated control group. But when administration of morphine

(5 mg/kg; i.p.) twice daily for a period of 5 days and discontinue the morphine on 6th day and observed the spontaneous withdrawal syndrome to morphine naloxone group in mice decreased the no of entries in open arm, as it showed that animals feel more anxiety as reflected by a significant ($p<0.01$) increase in anxiety behavioral of mice, when compared to morphine *per se* treated group.

The anxiety behavior seen due to termination of morphine and seen the spontaneous withdrawal symptoms which were significantly and dose dependently increased in the memantine (10 mg/kg; p.o.) ($p<0.01$), pirfenidone (200 mg/kg; p.o.) ($p<0.05$), piperine (10 mg/kg; p.o.) ($p<0.001$), piperine (15 mg/kg; p.o.) ($p<0.05$) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) ($p<0.01$) treated groups, when compared to morphine withdrawal group. The prime effect was observed only in pirfenidone (300 mg/kg; p.o.) ($p<0.01$) better than the standard memantine treated group pirfenidone (200 mg/kg) and piperine (10 and 15 mg/kg) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.). Thus, the high dose of pirfenidone (300 mg/kg; p.o) attenuated the morphine withdrawal symptoms as depicted by anxiety behavior in morphine dependent mice.

Table 23: Effect of pirfenidone and piperine on anxiety no. of entries in the open arm activity

Group no.	Group name	No. of entries in open arm Mean \pm SD
1.	Control	8.50 \pm 0.54
2.	Pirfenidone + Piperine	8.83 \pm 0.55
3.	Morphine <i>per se</i>	7.83 \pm 0.69
4.	Morphine Withdrawal	3.85 \pm 0.31 ^{***}
5.	Morphine + Memantine	5.63 \pm 0.53 ^{###}
6.	Morphine + Pirfenidone low dose	7.33 \pm 0.37 ^{###, ^}
7.	Morphine + Pirfenidone high dose	7.33 \pm 0.35 ^{###, ^}

8.	Morphine + Piperine low dose	6.67±0.41 ^{###,^^}
9.	Morphine + Piperine high dose	7.23±0.35 ^{###,^^}
10.	Morphine + Pirfenidone + Piperine low dose	7.99±0.23 ^{###,^^^}

***p<0.001 represent significant difference in group 3 vs group 4; ^{###}p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; [^]p<0.05, ^{^^}p<0.01, ^{^^^}p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way analysis of variance come succeed by Tukey's comparison test.

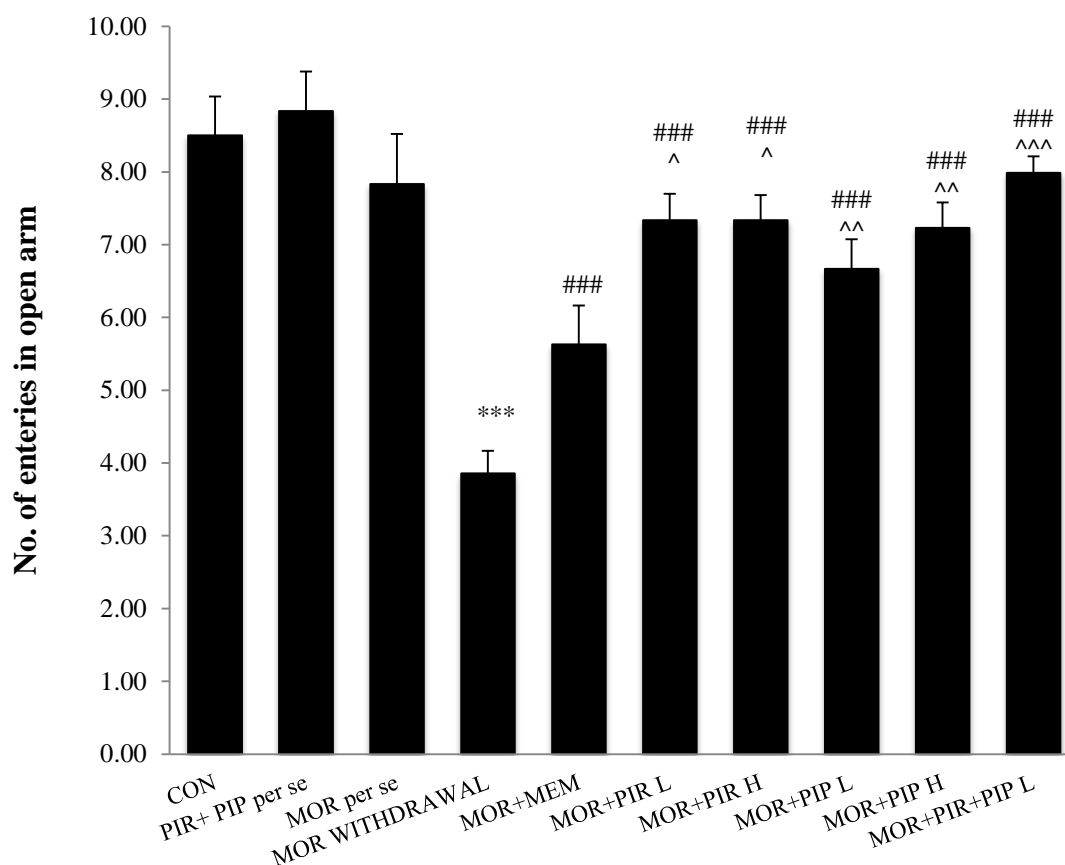


Fig. 25: Effect of pirfenidone and piperine on anxiety no. of entries in open arm activity.

Data represented as mean ± SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose.

***p<0.001 represent significant difference in group 3 vs group 4; ^{###}p<0.001 represent

significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; $\hat{p}<0.05$, $\hat{\hat{p}}<0.01$, $\hat{\hat{\hat{p}}}<0.001$ represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.2.9. Effect of pirfenidone and piperine on anxiety time spent in the open arm activity

It was observed that when administration of morphine *per se* to group 3 and pirfenidone + piperine to group 2 mice for 5 consecutive days, animals spent more time in open arm of elevated plus maze apparatus means they did not showed any significant change in anxiety behavior, as compared to vehicle treated control group. But when administration of morphine (5mg/kg; i.p.) twice daily for a period of 5 days and discontinue the morphine on 6th day and observed the spontaneous withdrawal syndrome to group 4 mice spent less time in open arm and more time in closed arm, as it showed that animals feel more anxiety as reflected by a significant ($p<0.001$) increase in anxiety behavioral of mice, when compared to morphine *per se* treated group.

The anxiety behavior seen due to termination of morphine and seen the spontaneous withdrawal symptoms which were significantly ($p<0.01$) and dose dependently reduced in the memantine (10 mg/kg; p.o.) ($p<0.01$), pirfenidone (300 mg/kg; p.o.) ($p<0.001$), piperine (10 mg/kg; p.o.) ($p<0.01$) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) ($p<0.001$) treated groups, when compared to morphine withdrawal group. The supreme effect was observed in combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups which was significantly ($p<0.05$) better than the standard memantine treated group pirfenidone (200 and 300 mg/kg) and piperine (10 and 15 mg/kg). Thus, this combination treatment attenuated the morphine withdrawal symptoms as depicted by anxiety behavior in morphine dependent mice.

Table 24: Effect of pirfenidone and piperine on anxiety time spent in the open arm activity

Group no.	Group name	Time spent in open arm Mean \pm SD
1.	Control	133.83 \pm 5.23
2.	Pirfenidone + Piperine	135.17 \pm 5.96
3.	Morphine <i>per se</i>	125.33 \pm 5.42
4.	Morphine Withdrawal	95.83 \pm 6.12 ^{***}
5.	Morphine + Memantine	116.56 \pm 6.66 ^{##}
6.	Morphine + Pirfenidone low dose	120.34 \pm 5.70
7.	Morphine + Pirfenidone high dose	129.17 \pm 6.11 ^{###,^}
8.	Morphine + Piperine low dose	111.22 \pm 5.76 [#]
9.	Morphine + Piperine high dose	118.10 \pm 7.76 ^{###}
10.	Morphine + Pirfenidone + Piperine low dose	134.50 \pm 7.12 ^{###,^^}

***p<0.001 represent significant difference ingroup 3 vs group 4; #p<0.05, ##p<0.01, ###p<0.001 represent significant difference ingroup 4 vs group 5, 6, 7, 8, 9, 10; ^p<0.05, ^^p<0.001 represent significant difference ingroup 5 vs 6, 7, 8, 9, 10 one-way analysis of variance come succeed by Tukey's comparison test.

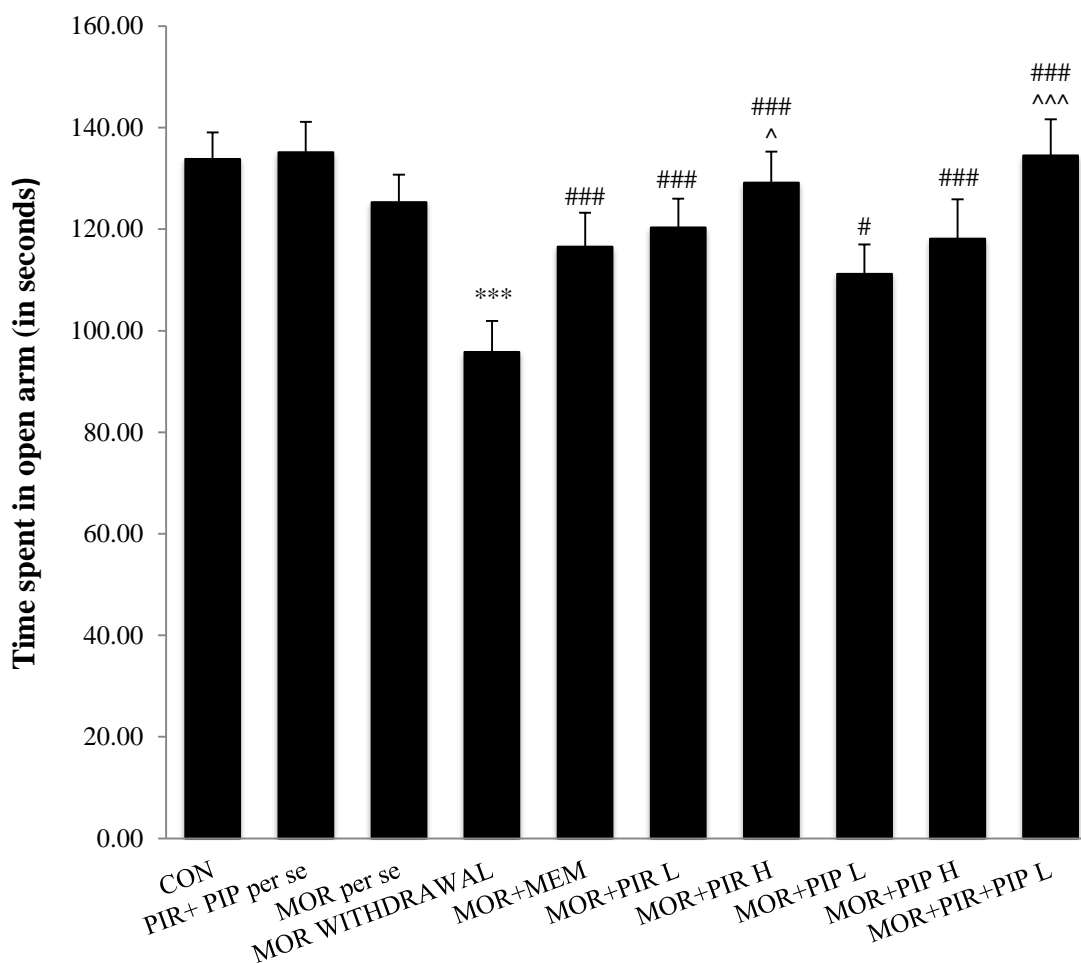


Fig. 26 : Effect of pirfenidone and piperine on anxiety time spent in the open arm activity

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. *** $p < 0.001$ represent significant difference in group 3 vs group 4; ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^ $p < 0.05$, ^^ $p < 0.01$, ^^ $p < 0.001$ represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.2.10. Effect of pirfenidone and piperine on analgesic MPE % activity

Pretreatment of morphine *per se* to morphine *per se* group and pirfenidone + piperine to pirfenidone-piperine *per se* group in mice for 5 consecutive days produced analgesic effect as assessed using the tail flick method in mice, showed

significant change in morphine *per se* and pirfenidone + piperine *per se* ($p < 0.001$) as compared to vehicle treated control group. But when administration of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days and discontinue the morphine on 6th day and observed the spontaneous withdrawal syndrome to morphine naloxone *per se* group in mice as reflected by a significant ($p < 0.001$) decreased in maximal percentage effect (MPE%) of mice, when compared to morphine *per se* treated group.

The analgesic behavior seen due to termination of morphine and seen the spontaneous withdrawal symptoms which were significantly ($p < 0.01$) and dose dependently increased in the memantine (10 mg/kg; p.o.) ($p < 0.01$) pirfenidone (300 mg/kg; p.o.) ($p < 0.05$) piperine (15 mg/kg; p.o.) ($p < 0.01$) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) ($p < 0.001$) treated groups, when compared to morphine withdrawal group. The foremost effect was observed in combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o) treated groups which was significantly ($p < 0.001$) better than the standard memantine treated group pirfenidone (200 and 300 mg/kg) and piperine (10 and 15 mg/kg). Thus, this combination treatment attenuated the morphine withdrawal symptoms as depicted by increased MPE% behavior in morphine dependent mice.

Table 25: Effect of pirfenidone and piperine on analgesic MPE % activity

Group no.	Group name	MPE% Mean \pm SD
1.	Control	6.33 \pm 1.03
2.	Pirfenidone + Piperine	55.17 \pm 4.85 ^{\$\$\$}
3.	Morphine <i>per se</i>	63.50 \pm 4.32 ^{\$\$\$}
4.	Morphine Withdrawal	32.33 \pm 3.20 ^{***}
5.	Morphine + Memantine	43.50 \pm 4.81 ^{##}
6.	Morphine + Pirfenidone low dose	37.83 \pm 4.12 ^{###}

7.	Morphine + Pirfenidone high dose	46.00±4.75 ^{###}
8.	Morphine + Piperine low dose	41.83±4.49 ^{###}
9.	Morphine + Piperine high dose	48.17±4.54 ^{###}
10.	Morphine + Pirfenidone + Piperine low dose	58.17±4.20 ^{###, ^^}

^{\$\$\$}p<0.001 represent significant difference ingroup 1 vs. group 2 and 3; ^{***}p<0.001 represent significant difference ingroup 3 vs group 4; [#]p<0.01, ^{###}p<0.001 represent significant difference ingroup 4 vs group 5, 6, 7, 8, 9, 10; ^{^^}p<0.001 represent significant difference ingroup 5 vs 6, 7, 8, 9, 10 one-way analysis of variance come succeed by Tukey's comparison test

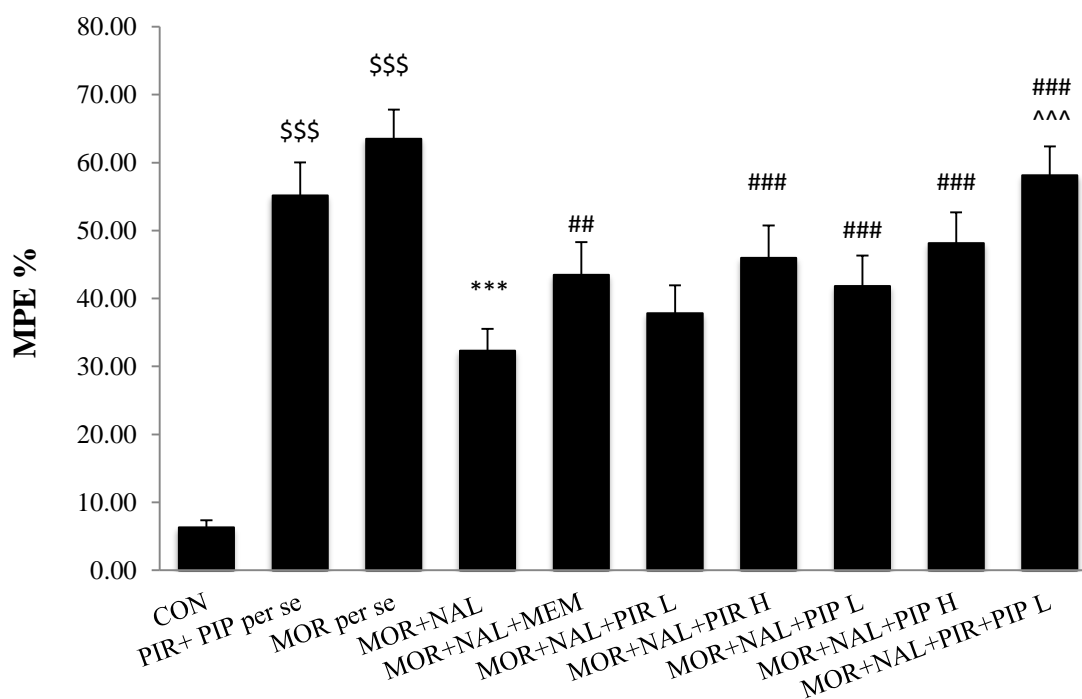


Fig. 27: Effect of pirfenidone and piperine on analgesic MPE % activity

Data represented as mean ± SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. ^{\$\$\$}p<0.001 represent significant difference in group 1 vs. group 2 and 3; ^{***}p<0.001 represent significant difference in group 3 vs group 4; [#]p<0.01, ^{###}p<0.001 reveals

main variation in group 4 vs group 5, 6, 7, 8, 9, 10; ^{^^}p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.2.11. Effect of pirfenidone and piperine on Defecation frequency

It was observed that, no significant change in defecation of mice frequency when administration of morphine *per se* to morphine *per se* group and pirfenidone + piperine to pirfenidone-piperine *per se* group in mice mice for 5 consecutive days as compared to vehicle treated control group. When administration of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days and discontinue the morphine on 6th day and observed the spontaneous withdrawal syndrome to morphine naloxone group in mice increased in the withdrawal syndrome, as reflected by a significant (p<0.001) increase the frequency of defecation in mice, when compared to morphine *per se* treated group.

Increased in defecation frequency had been seen due to termination of morphine and seen the spontaneous withdrawal symptoms which were significantly (p<0.001) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine withdrawal group. The foremost effect was observed in combination treated groups which was significantly (p<0.01) better than the standard memantine treated group, pirfenidone (200 and 300 mg/kg) and piperine (10 and 15 mg/kg). Thus, this combination treatment attenuated the morphine withdrawal symptoms as depicted by reduced defecation frequency in morphine dependent mice.

Table 26: Effect of pirfenidone and piperine on Defecation frequency

Group no.	Group name	Defecation frequency Mean \pm SD
1.	Control	1.17 \pm 0.10
2.	Pirfenidone + Piperine	1.50 \pm 0.12

3.	Morphine <i>per se</i>	1.83±0.11
4.	Morphine Withdrawal	2.83±0.11 ^{**}
5.	Morphine + Memantine	1.83±0.14 ^{##}
6.	Morphine + Pirfenidone low dose	1.67±0.11 ^{##}
7.	Morphine + Pirfenidone high dose	1.83±0.13 ^{##}
8.	Morphine + Piperine low dose	1.67±0.11 ^{##}
9.	Morphine + Piperine high dose	1.26±0.11 ^{###,^^}
10.	Morphine + Pirfenidone + Piperine low dose	1.00±0.13 ^{###,^^}

^{**}p<0.01, represent significant difference in group 3 vs group 4; ^{##}p<0.01, ^{###}p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^{^^}p<0.01, ^{^^^}p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way analysis of variance come succeed by Tukey's comparison test.

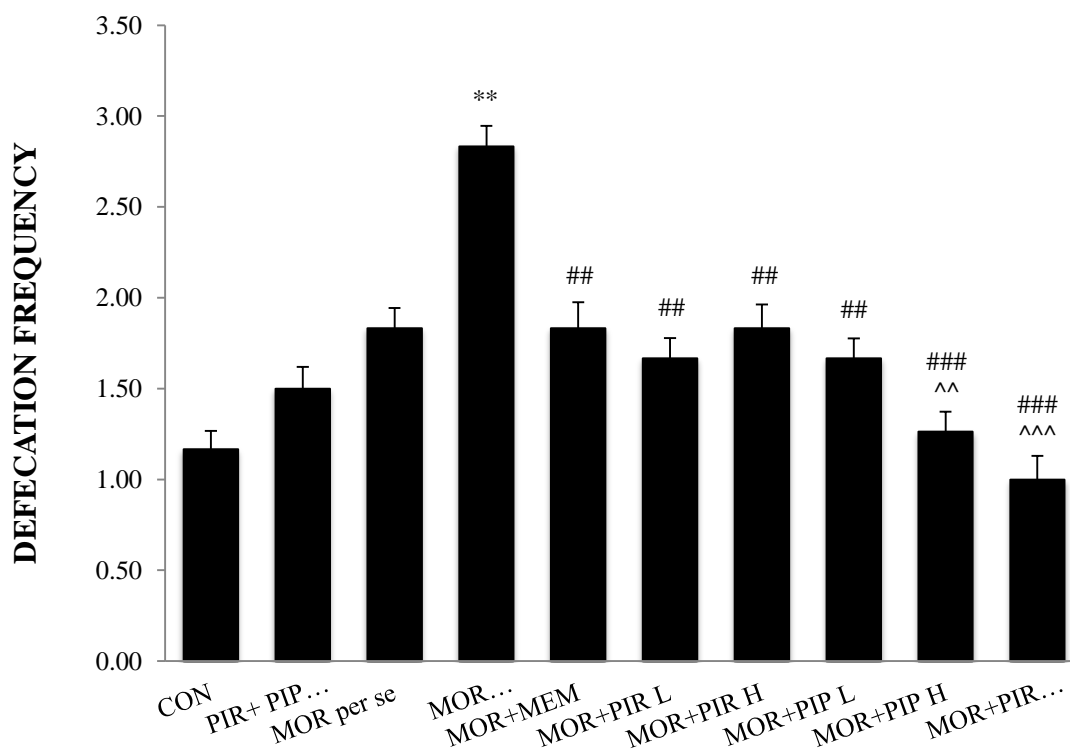


Fig. 28: Effect of pirfenidone and piperine on Defecation frequency

Data represented as mean \pm standard deviation were statistically examined using one-way Analysis of variance ANALYSIS OF VARIANCE come succeed by Tukey's comparison test. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine ; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. $^{\$}$ p<0.05, $^{\$\$}$ p<0.01, $^{\$\$\$}$ p<0.001 represent significant difference in group 1 vs group 2 and 3; * p<0.05, ** p<0.01, *** p<0.001 represent significant difference in group 3 vs group 4; $\#$ p<0.05, $\#\#$ p<0.01, $\#\#\#$ p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; $^{\wedge}$ p<0.05, $^{\wedge\wedge}$ p<0.01, $^{\wedge\wedge\wedge}$ p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.2.12. Effect of pirfenidone and piperine on urination frequency

It has been seen that, administration of morphine *per se* to morphine *per se* group 3 and pirfenidone + piperine to pirfenidone-piperine *per se* group in mice mice for 5 consecutive days did not showed any significant change in mice urination frequency, as compared to vehicle treated control group. But when administration of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days and discontinue the morphine on 6th day and observed the spontaneous withdrawal syndrome to morphine naloxone group in mice increased in the withdrawal syndrome as reflected by a significant (p<0.001) increase in urination of mice, when compared to morphine *per se* treated group.

Increased in urination seen due to termination of morphine and seen the spontaneous withdrawal symptoms which were significantly (p<0.001) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone-200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine withdrawal group. The best effect was observed in combination treated groups which was significantly (p<0.001) better than the standard memantine treated group, pirfenidone (200 and 300 mg/kg) and piperine (10 and 15 mg/kg). Thus, the combination of both i.e pirfenidone and piperine (200 and 10 mg/kg respectively) treatment attenuated the morphine withdrawal symptoms as depicted by reduced urine frequency in morphine dependent mice.

Table 27: Effect of pirfenidone and piperine on urination frequency

Group no.	Group name	Urine frequency Mean \pm SD
1.	Control	0.17 \pm 0.04
2.	Pirfenidone + Piperine	0.33 \pm 0.07
3.	Morphine <i>per se</i>	0.33 \pm 0.06
4.	Morphine + Naloxone	4.50 \pm 4.50 ^{**}
5.	Morphine + Naloxone+ Memantine	3.33 \pm 3.33 ^{##}
6.	Morphine + Naloxone+ Pirfenidone low dose	3.67 \pm 3.67 ^{##}
7.	Morphine + Naloxone+ Pirfenidone high dose	3.50 \pm 3.50 ^{##}
8.	Morphine + Naloxone+ Piperine low dose	3.17 \pm 3.17 ^{##}
9.	Morphine + Naloxone+ Piperine high dose	3.67 \pm 3.67 ^{###,^^}
10.	Morphine + Naloxone+ Pirfenidone + Piperine low dose	2.17 \pm 2.17 ^{###,^^}

^{**} p<0.01, represent significant difference in group 3 vs group 4; ^{##} p<0.01, ^{###} p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^{^^} p<0.01, ^{^^^} p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way analysis of variance come succeed by tukey's comparison test.

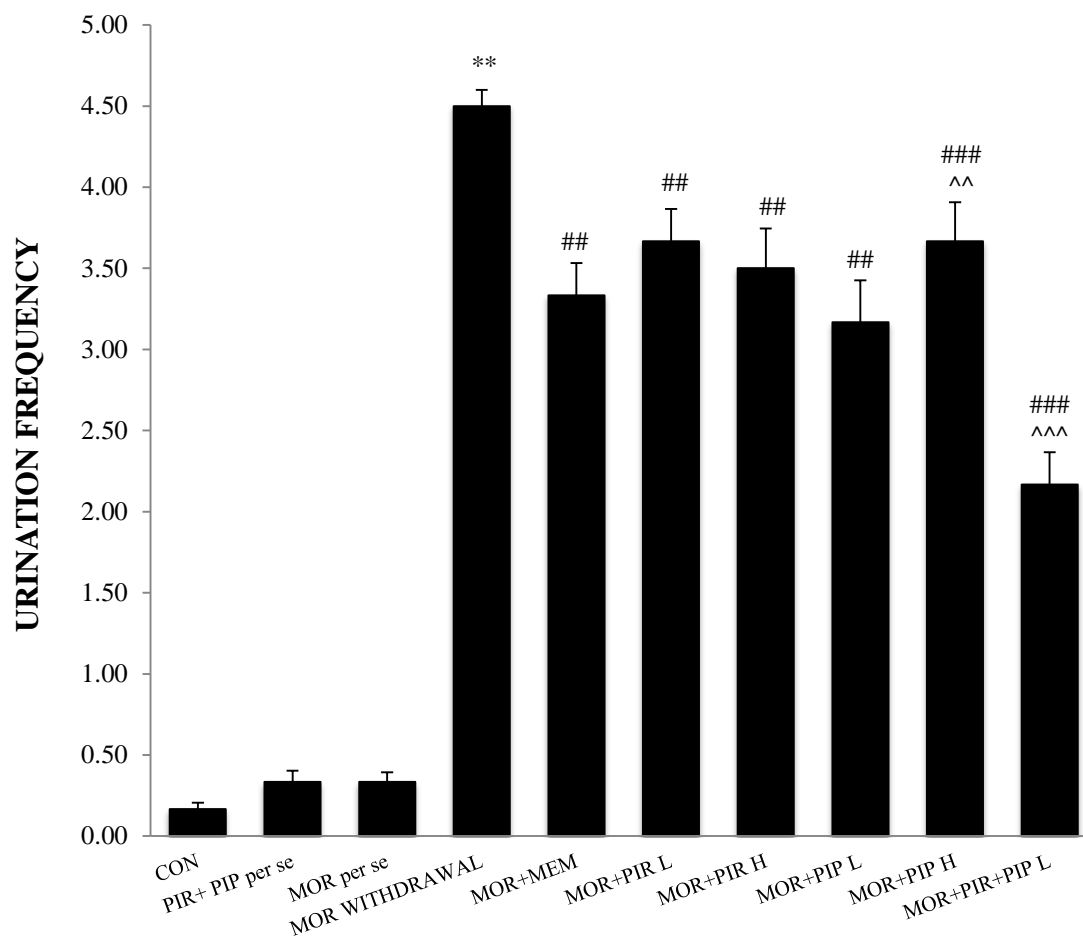


Fig. 29: Effect of pirfenidone and piperine on urination frequency

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose.

** $p < 0.01$ represent significant difference in group 3 vs group 4; ## $p < 0.01$, ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^^ $p < 0.01$, ^^ $p < 0.001$ represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.3. Biochemical assessments of precipitated animal model

5.1.3.1. Effect on tumor necrosis factor- α on precipitated animal model

It was observed that when pirfenidone + piperine administered to pirfenidone-piperine *per se* group and morphine *per se* to morphine *per se* group in mice for 5 consecutive days. There was no change in brain tissues of mice as

compared to vehicle treated control group. Administration of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days followed by a single injection of naloxone (8 mg/kg; i.p.) 2 hours after morphine injection to group 4 mice as reflected by a significantly ($p < 0.001$) increase on brain levels of TNF- α when compared to morphine *per se* treated group.

The increased level of TNF- α on brain tissues seen due to precipitation of naloxone-induced morphine withdrawal symptoms were significantly ($p < 0.001$) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone-200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine-naloxone treated group.

The best effect was observed in piperine (15 mg/kg; p.o) ($p < 0.05$), pirfenidone (300 mg/kg; p.o) ($p < 0.01$) and combination treated groups which was significantly ($p < 0.001$) better than the standard memantine treated group. Thus, this combination treatment significantly enhanced the inhibitory effect of treatment drugs on brain levels of TNF- α .

Table 28: Effect on tumor necrosis factor- α on precipitated animal model

Group no.	Group name	TUMOR NECROSIS FACTOR- α Mean \pm SD
1.	Control	100.00 \pm 10.00
2.	Pirfenidone + Piperine	60.52 \pm 6.04
3.	Morphine + Naloxone	290.12 \pm 25.32 ^{***}
4.	Morphine + Naloxone+ Memantine	243.34 \pm 24.33 ^{###}
5.	Morphine + Naloxone+ Pirfenidone low dose	220.12 \pm 22.01 ^{###}
6.	Morphine + Naloxone+ Pirfenidone high dose	215.1518.13 ^{###}
7.	Morphine + Naloxone+ Piperine low dose	200.36 \pm 20.02 ^{###}

8.	Morphine + Naloxone+ Piperine high dose	196.00±13.01 ^{###}
9.	Morphine + Naloxone+ Pirfenidone + Piperine low dose	110.26±11.03 ^{###, ^^}

^{\$}p<0.05, ^{\$\$}p<0.01, ^{\$\$\$}p<0.001 represent significant difference in group 1 vs. group 2 and 3; *p<0.05, **p<0.01, ***p<0.001 represent significant difference in group 3 vs group 4; #p<0.05, ##p<0.01, ###p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^p<0.05, ^^p<0.01, ^^p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way analysis of variance come succed by Tukey's comparson test

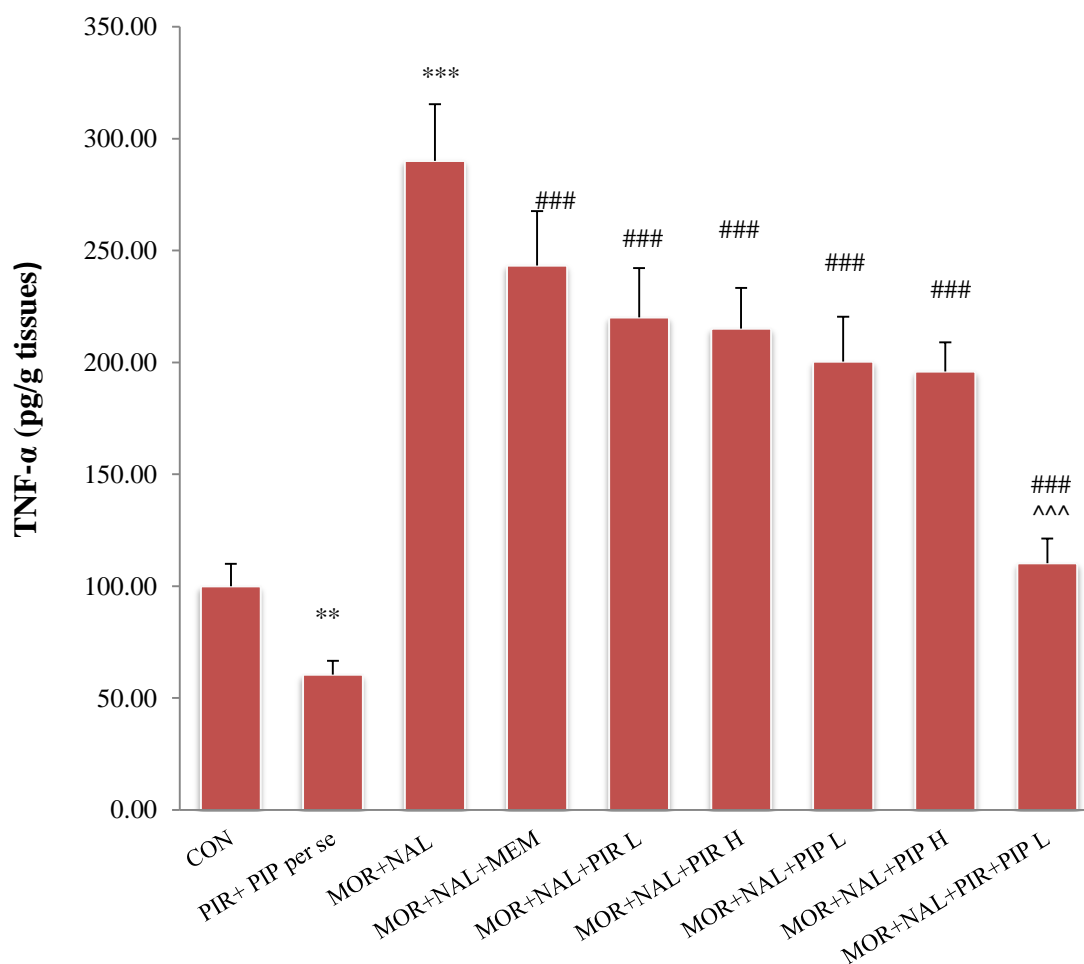


Fig. 30: Effect on TUMOR NECROSIS FACTOR- α on precipitated animal model

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. $^{\$}$ p<0.05, $^{\$\$}$ p<0.01, $^{\$ \$ \$}$ p<0.001 represent significant difference in group 1 vs. group 2 and 3; ** p<0.01, *** p<0.001 represent significant difference in group 3 vs group 4; $^{\#\#\#}$ p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; $^{\wedge\wedge}$ p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.3.2. Effect on GSH on precipitated animal model

The level of GSH in the mid brain regions were decreased significantly in when pirfenidone + piperine to pirfenidone-piperine *per se* group and morphine *per se* to morphine *per se* group in mice, when treated for 5 consecutive days, There was no change in brain tissues of mice as compared to vehicle treated control group. Treated of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days followed by a single injection of naloxone (8 mg/kg; i.p.) 2 hours after morphine injection to morphine naloxone group in mice as reflected by a significantly (p<0.001) increase on brain levels of GSH when compared to morphine *per se* treated group.

The increased level of GSH on brain tissues seen due to precipitation of naloxone-induced morphine withdrawal symptoms which were significantly (p<0.001) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone - 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine-naloxone treated group.

The best effect was observed in piperine (15 mg/kg; p.o) (p<0.05) and combination treated groups which was significantly (p<0.001) better than the standard memantine treated group. Thus, this combination treatment significantly enhanced the inhibitory effect of treatment drugs on brain levels of GSH.

Table 29: Effect on GSH on precipitated animal model

Group no.	Group name	GSH Mean \pm SD
1.	Control	5.70 \pm 0.16
2.	Pirfenidone + Piperine	8.74 \pm 0.36
3.	Morphine + Naloxone	3.34 \pm 0.34 ^{***}
4.	Morphine + Naloxone+ Memantine	5.22 \pm 0.45 ^{###}
5.	Morphine + Naloxone+ Pirfenidone low dose	4.38 \pm 0.41 ^{###}
6.	Morphine + Naloxone+ Pirfenidone high dose	4.23 \pm 0.43 ^{###}
7.	Morphine + Naloxone+ Piperine low dose	4.56 \pm 0.41 ^{##}
8.	Morphine + Naloxone+ Piperine high dose	4.72 \pm 0.49 ^{###}
9.	Morphine + Naloxone+ Pirfenidone + Piperine low dose	5.90 \pm 0.40 ^{###, ^^}

***p<0.001 represent significant difference in group 3vs group 4, ##p<0.01, ###p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^^p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way analysis of variance come succeed by tukey's comparsion test.

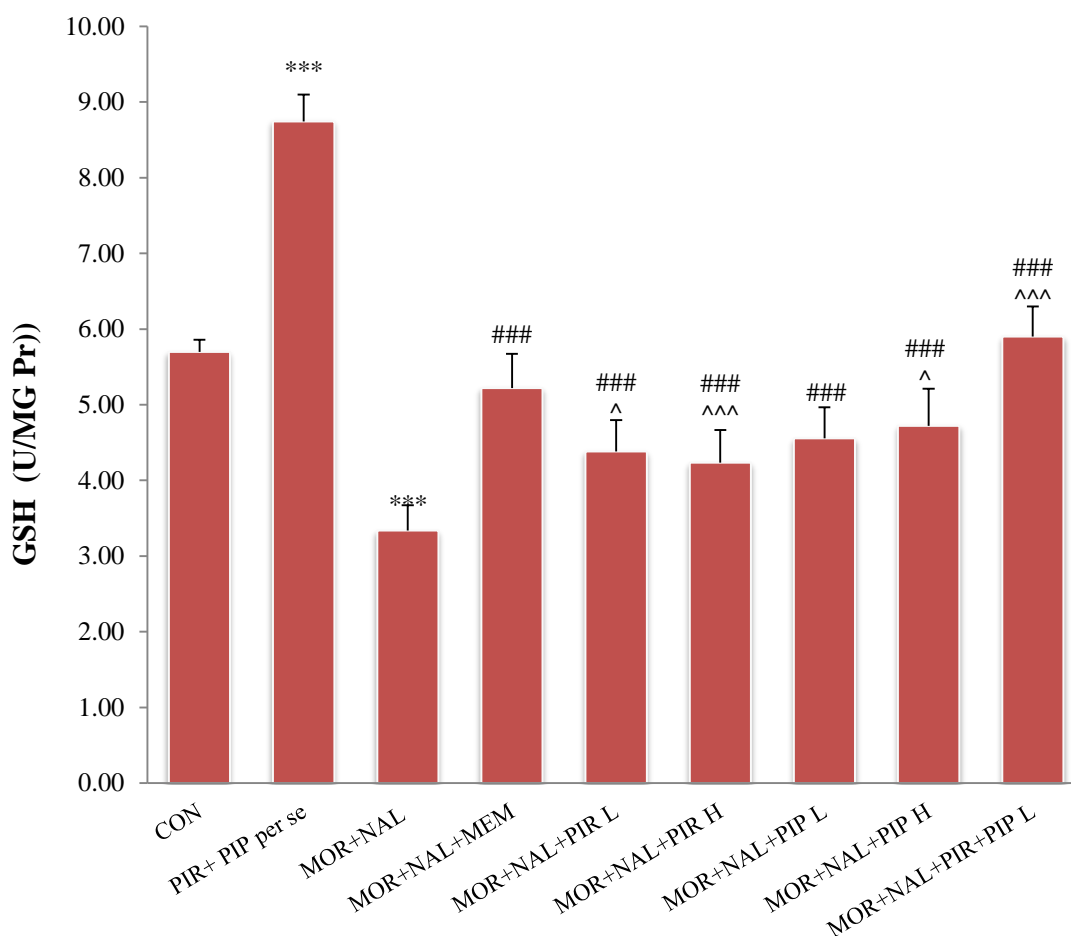


Fig. 31: Effect on GSH on precipitated animal model

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose.

*** $p < 0.001$ represent significant difference in group 3 vs division 4; ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^ $p < 0.05$, ^^ $p < 0.001$ represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.3.3. Effect on Catalase CAT on precipitated animal model

There was no change in the level of CAT in the mid brain regions of pirfenidone + piperine to pirfenidone-piperine *per se* group and morphine *per se* to morphine *per se* group in mice, when treated for 5 consecutive days, as compared to vehicle treated control group. Treated of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days followed by a single injection of naloxone (8 mg/kg; i.p.) 2 hours after

morphine injection to group 4 mice as reflected by a significantly ($p < 0.001$) increase on brain levels of CAT when compared to morphine *per se* treated group.

The increased level of CAT on brain tissues seen due to precipitation of naloxone-induced morphine withdrawal symptoms which were significantly ($p < 0.001$) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine-naloxone treated group.

The best effect was observed in combination treated groups which was significantly ($p < 0.001$) better than the standard memantine treated group. Thus, this combination treatment significantly enhanced the inhibitory effect of treatment drugs on brain levels of CAT.

Table 30: Effect on CAT on precipitated animal model

Group no.	Group name	CAT Mean \pm SD
1.	Control	5.56 \pm 0.19
2.	Pirfenidone + Piperine	8.96 \pm 0.23
3.	Morphine + Naloxone	2.13 \pm 0.27 ^{***}
4.	Morphine + Naloxone+ Memantine	5.10 \pm 0.27 ^{###}
5.	Morphine + Naloxone+ Pirfenidone low dose	4.25 \pm 0.22 ^{###}
6.	Morphine + Naloxone+ Pirfenidone high dose	4.10 \pm 0.24 ^{###}
7.	Morphine + Naloxone+ Piperine low dose	4.44 \pm 0.28 ^{###}
8.	Morphine + Naloxone+ Piperine high dose	4.65 \pm 0.26 ^{###,}
9.	Morphine + Naloxone+ Pirfenidone + Piperine low dose	6.00 \pm 0.25 ^{###, ^^}

*** $p < 0.001$ represent significant difference in group 3 vs group 4; ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^^ $p < 0.001$ represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way analysis of variance come succeed by tukey's comparison test.

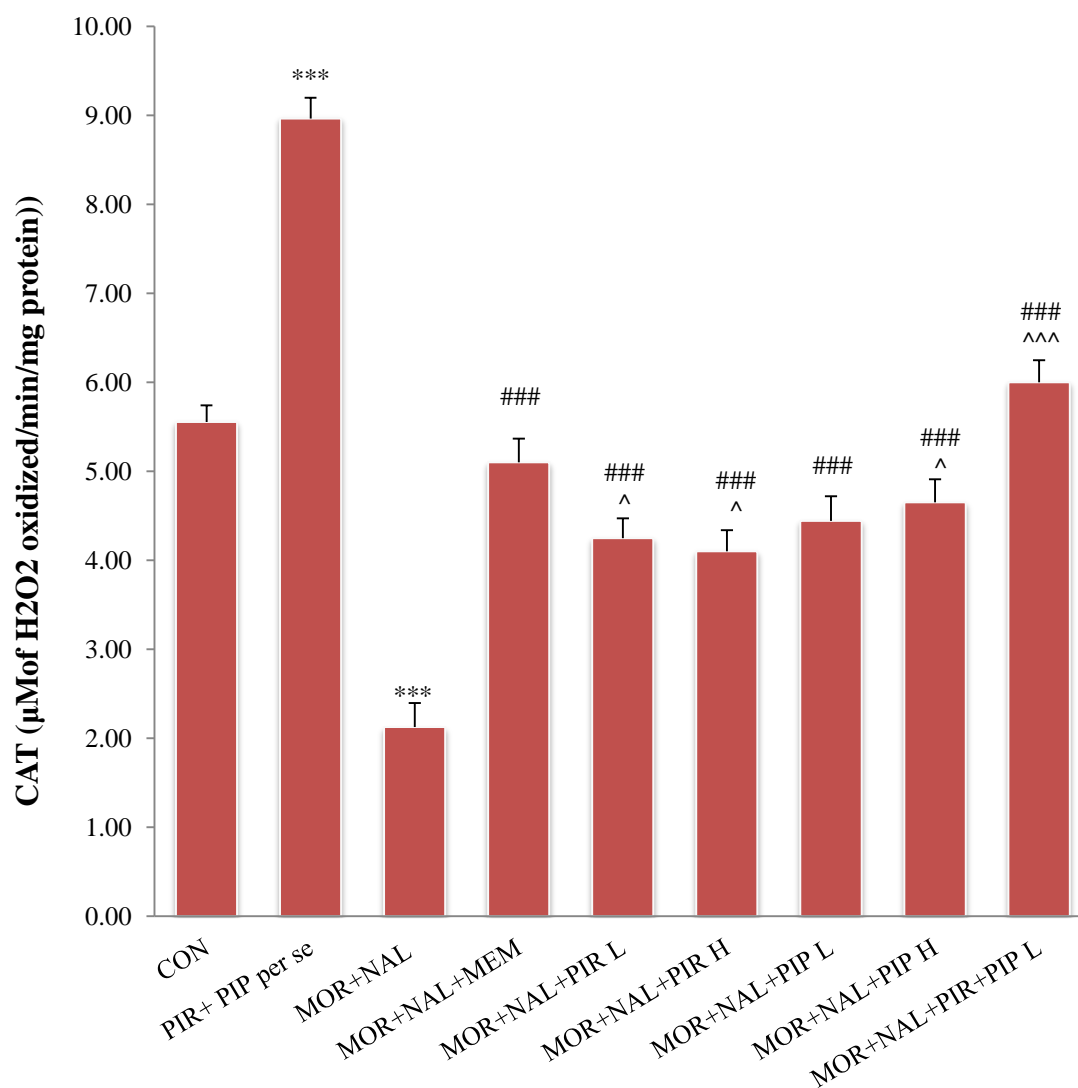


Fig. 32: Effect on CAT on precipitated animal model

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. *** $p < 0.001$ represents significant difference in group 3 vs group 4; ### $p < 0.001$ represents significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^^ $p < 0.001$ represents significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.3.4. Effect on SAG on precipitated animal model

There was no change in the level of SAG in the mid brain regions of pirfenidone + piperine to pirfenidone-piperine *per se* group and morphine *per se* to morphine *per se* group in mice, when treated for 5 consecutive days, as compared to vehicle treated control group. Treated with morphine (5 mg/kg; i.p.) twice daily for a period of 5 days followed by a single injection of naloxone (8 mg/kg; i.p.) 2 hours after morphine injection to morphine naloxone group in mice as reflected by a significantly ($p < 0.001$) increase on brain levels of CAT when compared to morphine *per se* treated group.

The increased level of SAG on brain tissues seen due to precipitation of naloxone-induced morphine withdrawal symptoms which were significantly ($p < 0.001$) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine-naloxone treated group.

The best effect was observed in combination treated groups which was significantly ($p < 0.001$) better than the standard memantine treated group. Thus, this combination treatment significantly enhanced the inhibitory effect of treatment drugs on brain levels of SAG.

Table 31: Effect on SAG on precipitated animal model

Group no.	Group name	SAG Mean \pm SD
1.	Control	3.21 \pm 1.47
2.	Pirfenidone + Piperine	1.50 \pm 1.60 ^{***}
3.	Morphine + Naloxone	8.92 \pm 1.36 ^{***}
4.	Morphine + Naloxone+ Memantine	7.52 \pm 1.78 ^{###}
5.	Morphine + Naloxone+ Pirfenidone low dose	7.77 \pm 1.36 ^{###}

6.	Morphine + Naloxone+ Pirfenidone high dose	7.41±2.19 ^{###}
7.	Morphine + Naloxone+ Piperine low dose	7.26±1.47 ^{###}
8.	Morphine + Naloxone+ Piperine high dose	7.01±1.47 ^{###}
9.	Morphine + Naloxone+ Pirfenidone + Piperine low dose	4.89±0.40 ^{###, ^^}

^{***}p<0.001 represent significant difference in group 3 vs group 4; ^{###}p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^{^^}p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way analysis of variance come succeed by tukey's comparison test.

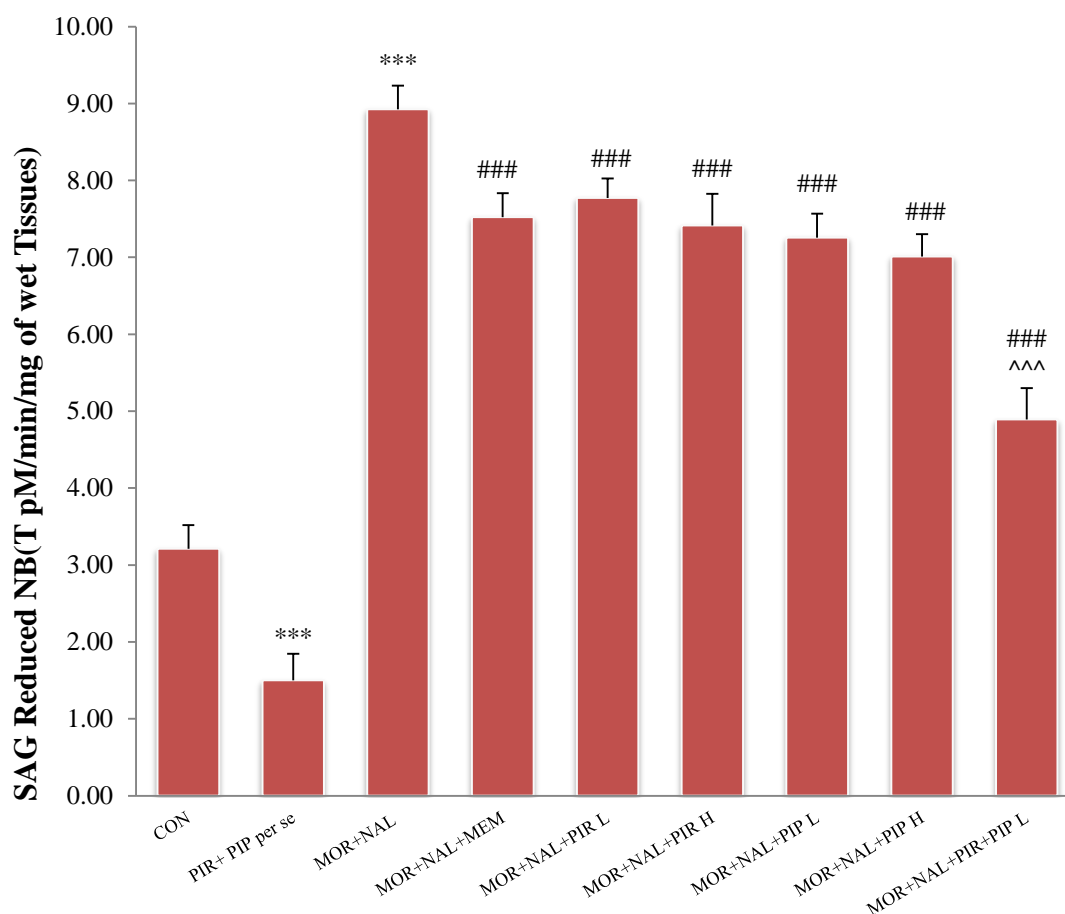


Fig. 33: Effect on SAG on precipitated animal model

Data represented as mean ± SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose.

*** $p < 0.001$ represent significant difference in group 3 vs group 4; ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^^ $p < 0.001$ represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.3.5. Effect on TBARS on precipitated animal model

There was no change in the level of TBARS in the mid brain regions of pirfenidone + piperine to pirfenidone-piperine *per se* group and morphine *per se* to morphine *per se* group in mice, when treated for 5 consecutive days, as compared to vehicle treated control group. Treated with morphine (5 mg/kg; i.p.) twice daily for a period of 5 days followed by a single injection of naloxone (8 mg/kg; i.p.) 2 hours after morphine injection to morphine naloxone group in mice as reflected by a significantly ($p < 0.001$) increase on brain levels of TBARS when compared to morphine *per se* treated group.

The increased level of TBARS on brain tissues seen due to precipitation of naloxone-induced morphine withdrawal symptoms which were significantly ($p < 0.001$) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine-naloxone treated group.

The best effect was observed in combination treated groups which was significantly ($p < 0.001$) better than the standard memantine treated group. Thus, this combination treatment significantly enhanced the inhibitory effect of treatment drugs on brain levels of TBARS

Table 32: Effect on TBARS on precipitated animal model

Group no.	Group name	TBARS Mean \pm SD
1.	Control	3.333 \pm 0.35
2.	Pirfenidone + Piperine	1.36 \pm 0.31 ^{***}
3.	Morphine + Naloxone	9.84 \pm 0.34 ^{***}
4.	Morphine + Naloxone+ Memantine	7.91 \pm 0.25 ^{###}
5.	Morphine + Naloxone+ Pirfenidone low dose	8.15 \pm 0.25 ^{###}
6.	Morphine + Naloxone+ Pirfenidone high dose	7.56 \pm 0.42 ^{###}
7.	Morphine + Naloxone+ Piperine low dose	6.12 \pm 0.27 ^{###,^}
8.	Morphine + Naloxone+ Piperine high dose	6.89 \pm 0.37 ^{###}
9.	Morphine + Naloxone+ Pirfenidone + Piperine low dose	4.64 \pm 0.33 ^{###,^^}

^{***}p<0.001 represent significant difference in group 3 vs group 4; ^{###}p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; [^]p<0.05, ^{^^}p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way analysis of variance come succeed by tukey's comparsion test

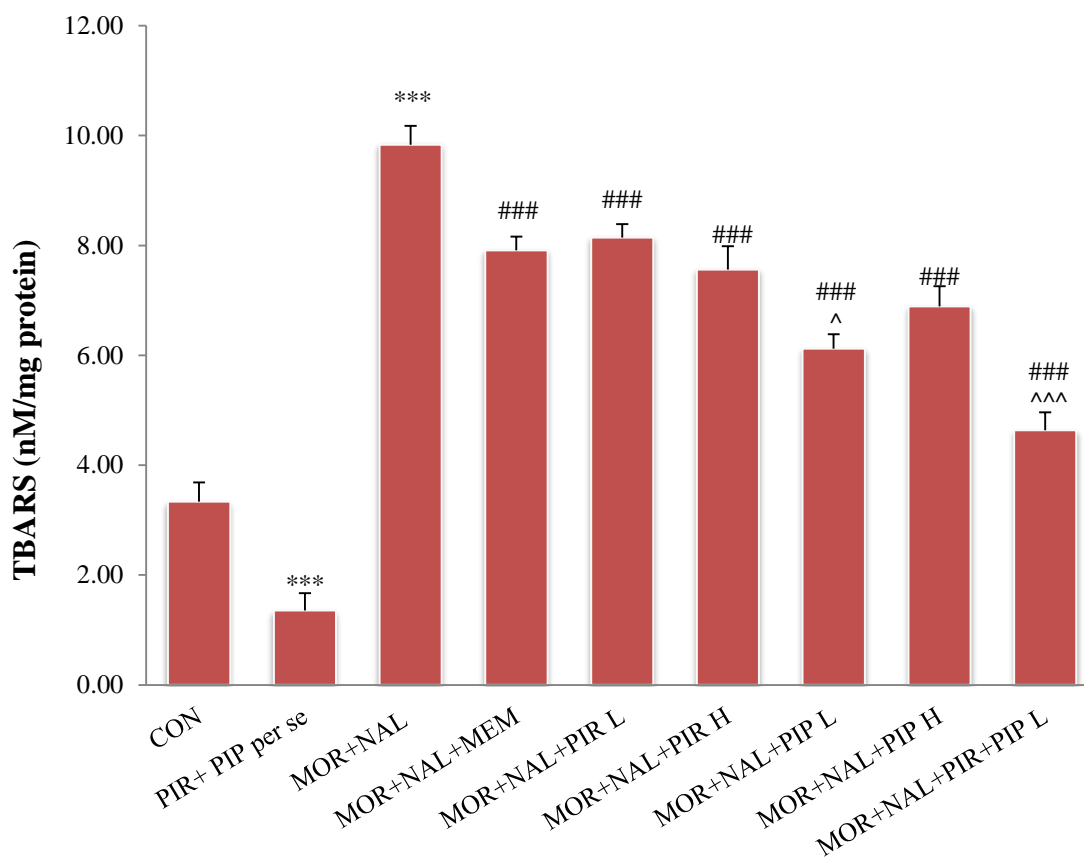


Fig. 34: Effect of TBARS on precipitated animal model

Data represented as mean \pm standard deviation were statistically examined using one-way analysis of variance (ANALYSIS OF VARIANCE) succeed by Tukey's multiple comparison test. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; MEM: Memantine; L: low dose; H: high dose. *** $p < 0.001$ represents significant difference in group 1 vs group 2, 3; ### $p < 0.001$ represents represents significant difference in group 3 vs group 4, 5, 6, 7, 8 and 9 ^^ $p < 0.001$ represents significant difference in group 5 vs 6, 7, 8 and 9.

5.1.4. Behavioural evaluations of spontaneous animal model

5.1.4.1. Effect of tumor necrosis factor- α on spontaneous animal model

It was observed that when pirfenidone + piperine administered to pirfenidone-piperine *per se* group and morphine *per se* to morphine *per se* group in mice for 5 consecutive days, There was no change in brain tissues of mice as

compared to vehicle treated control group. Administration of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days and discontinue the drug on 6th day, to morphine naloxone group in mice as reflected by a significantly ($p < 0.001$) increase on brain levels of TNF- α when compared to morphine *per se* treated group.

The increased level of TNF- α on brain tissues seen due to termination of morphine and were significantly ($p < 0.001$) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine-naloxone treated group.

The best effect was observed in piperine (15 mg/kg; p.o) ($p < 0.05$) and combination treated groups which was significantly ($p < 0.001$) better than the standard memantine treated group. Thus, this combination treatment significantly enhanced the inhibitory effect of treatment drugs on brain levels of TNF- α .

Table 33: Effect on tumor necrosis factor- α on spontaneous animal model

Group no.	Group name	TUMOR NECROSIS FACTOR- α Mean \pm SD
1.	Control	101.33 \pm 11.93
2.	Pirfenidone + Piperine	60.52 \pm 5.61
3.	Morphine withdrawal	309.32 \pm 17.36 ^{***}
4.	Morphine + Memantine	236.55 \pm 14.38 ^{###}
5.	Morphine + Pirfenidone low dose	211.37 \pm 15.68 ^{###}
6.	Morphine + Pirfenidone high dose	202.36 \pm 9.27 ^{###}
7.	Morphine + Piperine low dose	195.00 \pm 17.72 ^{###}
8.	Morphine + Piperine high dose	190.00 \pm 21.89 ^{###, ^}
9.	Morphine + Pirfenidone + Piperine low dose	132.20 \pm 9.20 ^{###, ^^}

*** $p < 0.001$ represent significant difference in group 3 vs group 4; ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^ $p < 0.05$, ^^ $p < 0.001$ represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way analysis of variance come succeed by Tukey's comparison test

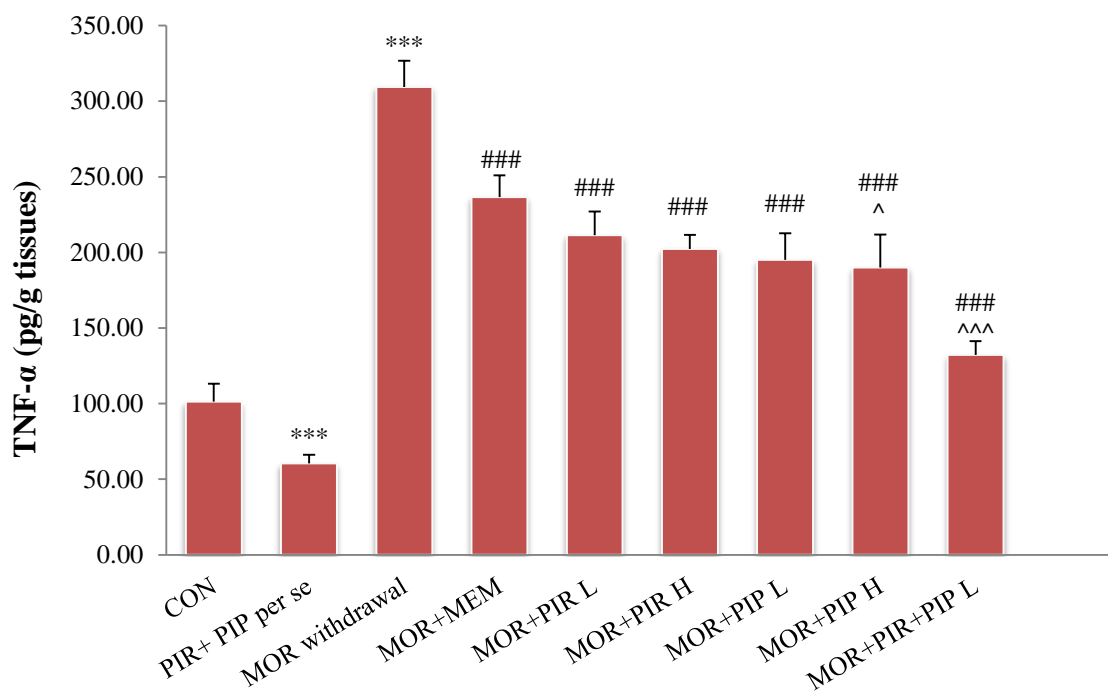


Fig. 35: Effect on tumor necrosis factor- α on spontaneous animal model

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. *** $p < 0.001$ represent significant difference in group 3 vs group 4; ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^ $p < 0.05$, ^^ $p < 0.001$ represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.4.2. Effect on GSH on spontaneous animal model

There was no change in the level of CAT in the mid brain regions of pirfenidone + piperine to pirfenidone-piperine *per se* group and morphine *per se* to morphine *per se* group in mice, when treated for 5 consecutive days, as compared to vehicle treated control group. Treated of morphine (5 mg/kg; i.p.) twice daily for a

period of 5 days and discontinue the drug on 6th day, to morphine naloxone group in mice as reflected by a significantly ($p<0.001$) increase on brain levels of GSH when compared to morphine *per se* treated group.

The increased level of GSH on brain tissues seen due to termination of morphine spontaneously which were significantly ($p<0.001$) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine-naloxone treated group.

The effect was observed in piperine (15 mg/kg; p.o) ($p<0.05$) and combination treated groups which was significantly ($p<0.001$) better than the standard memantine treated group. Thus, the best was in combination treatment significantly enhanced the inhibitory effect of treatment drugs on brain levels of GSH.

Table 34: Effect on GSH on spontaneous animal model

Group no.	Group name	GSH Mean \pm SD
1.	Control	5.21 \pm 0.19
2.	Pirfenidone + Piperine	8.53 \pm 0.32 ^{***}
3.	Morphine withdrawal	2.99 \pm 0.25 ^{***}
4.	Morphine + Memantine	5.35 \pm 0.26 ^{###}
5.	Morphine + Pirfenidone low dose	4.88 \pm 0.27 ^{###}
6.	Morphine + Pirfenidone high dose	4.80 \pm 0.24 ^{###}
7.	Morphine + Piperine low dose	3.78 \pm 0.25 ^{###}
8.	Morphine + Piperine high dose	4.00 \pm 0.26 ^{###}
9.	Morphine + Pirfenidone + Piperine low dose	5.78 \pm 0.21 ^{###, ^^}

*** $p < 0.001$ represent significant difference in group 3 vs group 4; ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^^ $p < 0.001$ represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way analysis of variance come succeed by Tukey's comparison test.

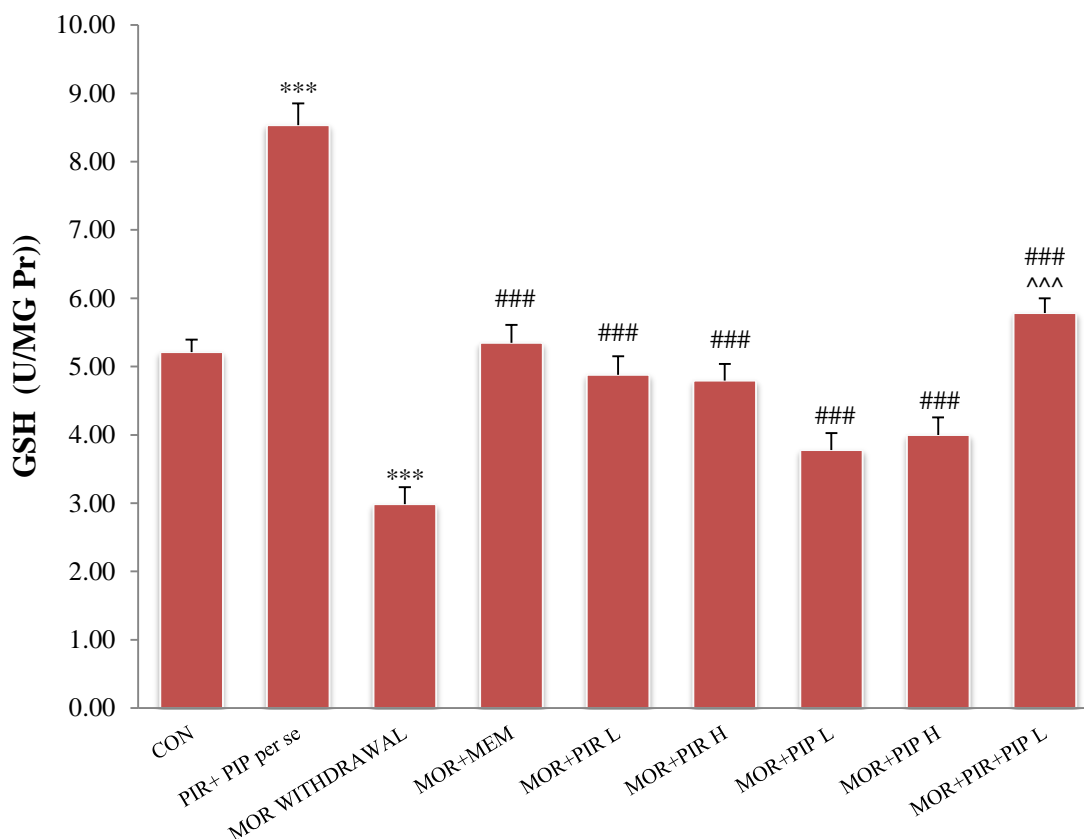


Fig. 36: Effect on GSH on spontaneous animal model

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose.

*** $p < 0.001$ represent significant difference in group 3 vs group 4; ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^^ $p < 0.001$ represent significant difference in group 5 vs 6, 7, 8, 9, 10.

5.1.4.3. Effect on Catalase (CAT) on spontaneous animal model

There was no change in the level of CAT in the mid brain regions of pirfenidone + piperine to pirfenidone-piperine *per se* group and morphine *per se* to

morphine *per se* group in mice, when treated for 5 consecutive days, as compared to vehicle treated control group. Treated of morphine (5 mg/kg; i.p.) twice daily for a period of 5 days followed by a single injection of naloxone (8 mg/kg; i.p.) 2 hours after morphine injection to group 4 mice as reflected by a significantly ($p<0.001$) increase on brain levels of CAT when compared to morphine *per se* treated group.

The increased level of CAT on brain tissues seen due to precipitation of naloxone-induced morphine withdrawal symptoms which were significantly ($p<0.001$) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine-naloxone treated group.

The best effect was observed in combination treated groups which was significantly ($p<0.001$) better than the standard memantine treated group. Thus, this combination treatment significantly enhanced the inhibitory effect of treatment drugs on brain levels of CAT

Table 35: Effect on CAT on spontaneous animal model

Group no.	Group name	CAT Mean \pm SD
1.	Control	5.340.18
2.	Pirfenidone + Piperine	7.99 \pm 0.32
3.	Morphine withdrawal	3.10 \pm 0.25 ^{***}
4.	Morphine + Memantine	5.00 \pm 0.37 ^{###}
5.	Morphine + Pirfenidone low dose	4.86 \pm 0.39 ^{###}
6.	Morphine + Pirfenidone high dose	4.75 \pm 0.35 ^{###}
7.	Morphine + Piperine low dose	4.77 \pm 0.39 ^{###}
8.	Morphine + Piperine high dose	4.99 \pm 0.37 ^{###} ,
9.	Morphine + Pirfenidone + Piperine low dose	6.23 \pm 0.33 ^{###, ^^}

*** $p < 0.001$ represent significant difference in group 3 vs group 4; ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^^ $p < 0.001$ represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way analysis of variance come after by Tukey's various prominent examination .

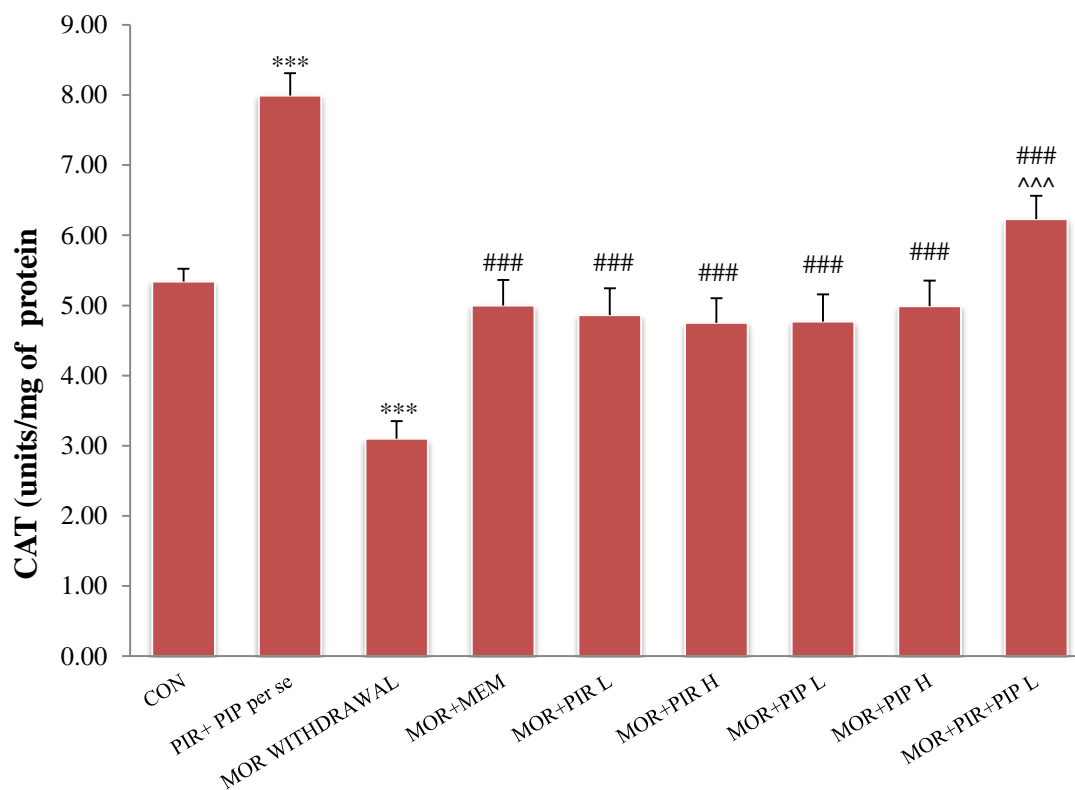


Fig. 37: Effect on CAT on spontaneous animal model

Data represented as mean \pm standard deviation were statistically identified by the utilization of one-way analysis of variance come after by Tukey's various prominent examinations. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine ; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$$$}p < 0.001$ represent significant difference in group 1 vs. group 2 and 3; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ represent significant difference in group 3 vs group 4; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ represent significant difference of group 5 vs 6, 7, 8, 9, 10.

5.1.4.4. Effect on SAG on spontaneous animal model

There was no change in the level of SAG in the mid brain regions of pirfenidone + piperine to pirfenidone-piperine *per se* group and morphine *per se* to morphine *per se* group in mice, when treated for 5 consecutive days, as compared to vehicle treated control group. Treated with morphine (5 mg/kg; i.p.) twice daily for a period of 5 days followed by a single injection of naloxone (8 mg/kg; i.p.) 2 hours after morphine injection to morphine naloxone group in mice as reflected by a significantly ($p < 0.001$) increase on brain levels of CAT when compared to morphine *per se* treated group.

The increased level of SAG on brain tissues seen due to precipitation of naloxone-induced morphine withdrawal symptoms which were significantly ($p < 0.001$) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirfenidone 200 mg/kg + piperine 10 mg/kg; p.o.) treated groups, when compared to morphine-naloxone treated group.

The best effect was observed in combination treated groups which was significantly ($p < 0.001$) better than the standard memantine treated group. Thus, this combination treatment significantly enhanced the inhibitory effect of treatment drugs on brain levels of SAG

Table 36: Effect on SAG on spontaneous animal model

Group no.	Group name	SAG Mean \pm SD
1.	Control	3.46 \pm 0.35
2.	Pirfenidone + Piperine	1.52 \pm 0.15 ^{***}
3.	Morphine + Naloxone	9.52 \pm 0.95 ^{***}
4.	Morphine + Naloxone+ Memantine	7.83 \pm 0.78 ^{###}
5.	Morphine + Naloxone+ Pirfenidone low dose	7.00 \pm 0.70 ^{###}

6.	Morphine + Naloxone+ Pirfenidone high dose	7.02±0.65 ^{###}
7.	Morphine + Naloxone+ Piperine low dose	7.12±0.63 ^{###}
8.	Morphine + Naloxone+ Piperine high dose	7.00±0.54 ^{###}
9.	Morphine + Naloxone+ Pirfenidone + Piperine low dose	4.00±0.48 ^{###, ^^}

^{***}p<0.001 represent significant difference in group 3 vs division 4; ^{###}p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^{^^}p<0.001 represent significant difference in group 5 vs 6, 7, 8, 9, 10 one-way analysis of variance follow by Tukey's various prominent examination.

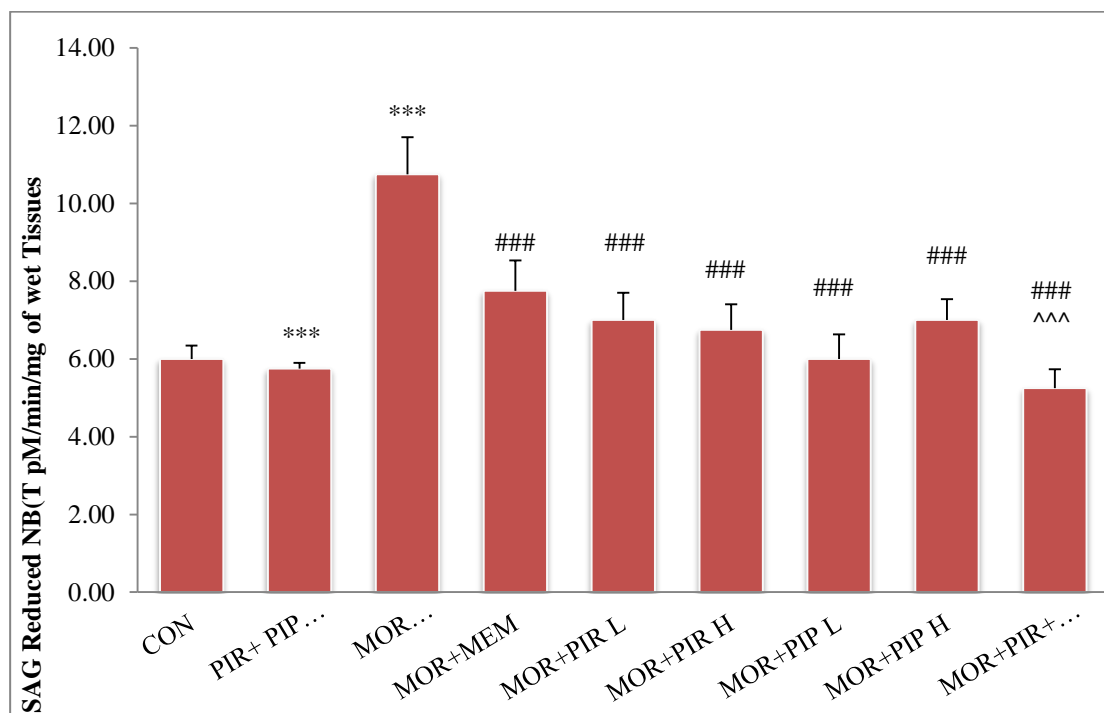


Fig. 38: Effect on SAG on spontaneous animal model

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose.

^{***}p<0.001 represent significant difference in group 3 vs group 4; ^{###}p<0. represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^{^^}p<0.001 represent significant difference indivision 5 vs 6, 7, 8, 9, 10.

5.1.4.5. Effect of TBARS on spontaneous animal model

There was no change in the level of TBARS in the mid brain regions of pirlfenidone + piperine to pirlfenidone piperine *per se* group and morphine *per se* to morphine *per se* group in mice, when treated for 5 consecutive days, as compared to vehicle treated control group. Treated with morphine (5 mg/kg; i.p.) twice daily for a period of 5 days followed by a single injection of naloxone (8 mg/kg; i.p.) 2 hours after morphine injection to group 4 mice as reflected by a significantly ($p < 0.001$) increase on brain levels of TBARS when compared to morphine *per se* treated group.

The increased level of TBARS on brain tissues seen due to precipitation of naloxone-induced morphine withdrawal symptoms which were significantly ($p < 0.001$) and dose dependently reduced in the memantine (10 mg/kg; p.o.), pirlfenidone (200 and 300 mg/kg; p.o.), piperine (10 and 15 mg/kg; p.o.) and combination (pirlfenidone- 200 mg/kg + piperine- 10 mg/kg; p.o.) treated groups, when compared to morphine-naloxone treated group.

The best effect was observed in combination treated groups which was significantly ($p < 0.001$) better than the standard memantine treated group. Thus, this combination treatment significantly enhanced the inhibitory effect of treatment drugs on brain levels of TBARS.

Table 37: Effect on TBARS on spontaneous animal model

Group no.	Group name	TBARS Mean \pm SD
1.	Control	3.50 \pm 0.35
2.	Pirfenidone + Piperine	1.34 \pm 0.33 ^{***}
3.	Morphine withdrawal	11.17 \pm 0.35 ^{***}
4.	Morphine + Memantine	7.33 \pm 0.52 ^{###}
5.	Morphine + Pirfenidone low dose	7.17 \pm 0.42 ^{###}
6.	Morphine + Pirfenidone high dose	7.23 \pm 0.49 ^{###}
7.	Morphine + Piperine low dose	7.10 \pm 0.36 ^{###}
8.	Morphine + Piperine high dose	7.23 \pm 0.40 ^{###}
9.	Morphine + Pirfenidone + Piperine low dose	4.67 \pm 0.32 ^{###, ^^}

^{***}p<0.001 represent significant difference in group 3 vs division 4; ^{###}p<0.001 represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^{^^}p<0.001 represent significant difference in division 5 vs 6, 7, 8, 9, 10 one-way analysis of variance succeed by Tukey's various prominent examination.

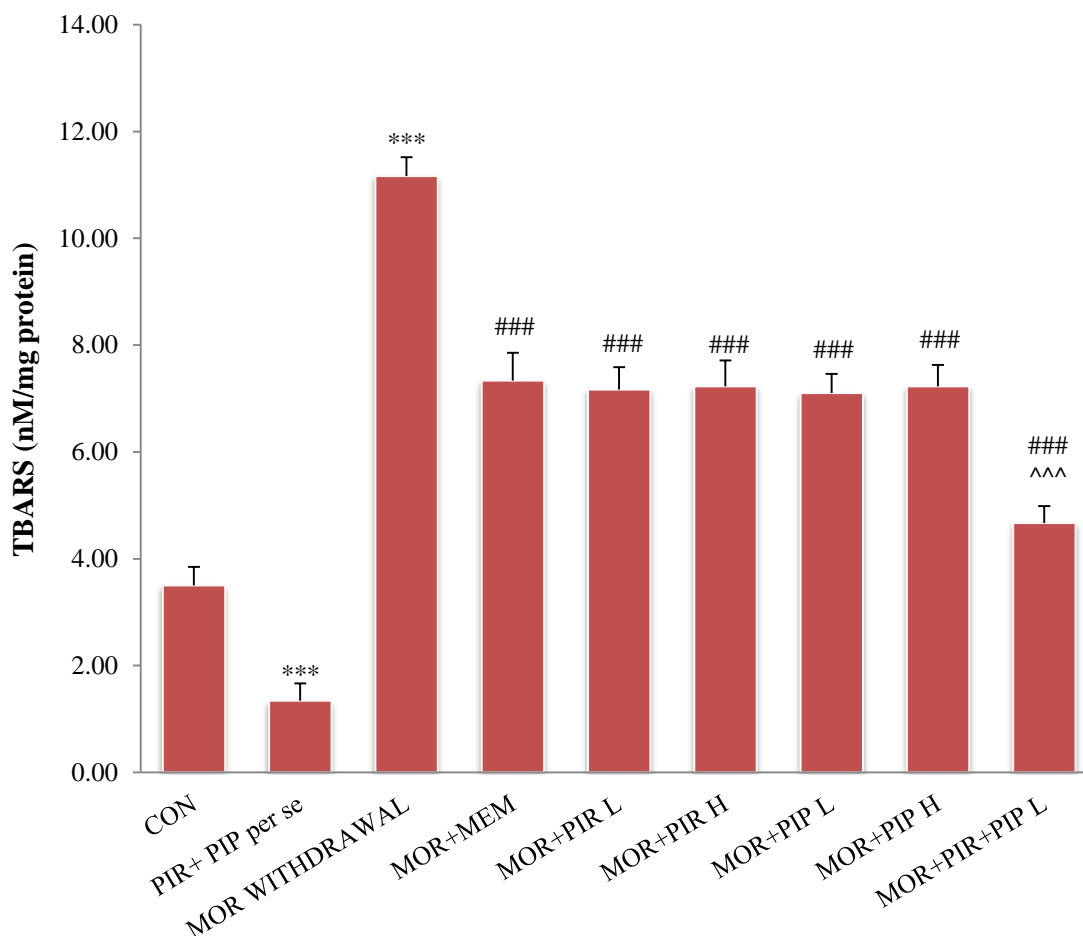


Fig. 39: Effect on TBARS on spontaneous animal model

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose.

*** $p < 0.001$ represent significant difference in group 3 vs group 4; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ represent significant difference in group 4 vs group 5, 6, 7, 8, 9, 10; ^^ $p < 0.001$ represent significant difference in division 5 vs 6, 7, 8, 9, 10.

5.2. *In vitro*

Action of pirfenidone and piperine on naloxone precipitated opioid withdrawal on contracture of rat ileum.

Naloxone challenge immediately after a brief period of 4-min morphine exposure elicited a strong contracture in the rat ileum preparation in terms of the tension ratio

results. Administration of pirfenidone significantly and dose dependently attenuated this naloxone-induced withdrawal response in morphine withdrawn rat ileum preparation as assessed in terms of tension ratio in morphine/naloxone group, when compared to that of control group. Further, the administration of piperine significantly and dose dependently attenuated naloxone-induced withdrawal response in morphine withdrawn rat ileum. Memantine which was taken as standard drug also attenuated the morphine withdrawal syndrome but not as good achieved in pirfenidone and piperine. The effect of each drug at a low dose was noted to be significantly lower than that of the drug at high dose. But the best result was attained by the combination of both the drugs i.e pirfenidone+piperine.

Table 38: In vitro Effect of pirfenidone and piperine and its combination

Group no.	Group name	In vitro Mean \pm SD
1.	Control	78.33 \pm 9.33
2.	Memantine	60.67 \pm 6.86 ^{\$\$}
3.	Pirfenidone low dose	56.83 \pm 9.91 ^{\$\$\$}
4.	Pirfenidone high dose	45.33 \pm 6.19 ^{\$\$\$} , ^{^^}
5.	Piperine low dose	51.33 \pm 5.35 ^{\$\$\$}
6.	Piperine high dose	36.67 \pm 4.89 ^{\$\$\$} , ^{^^}
7.	Pirfenidone + Piperine low dose	19.17 \pm 5.85 ^{\$\$\$} , ^{^^}

^{\$\$}p<0.01, ^{\$\$\$}p<0.001 represent significant difference in group 1 vs group 2, 3, 4, 5, 6, 7; ^{^^}p<0.01, ^{^^^}p<0.001 represent significant difference in division 2 vs division 3, 4, 5, 6, 7 one-way analysis of variance succed by Tukey's various prominent examination.

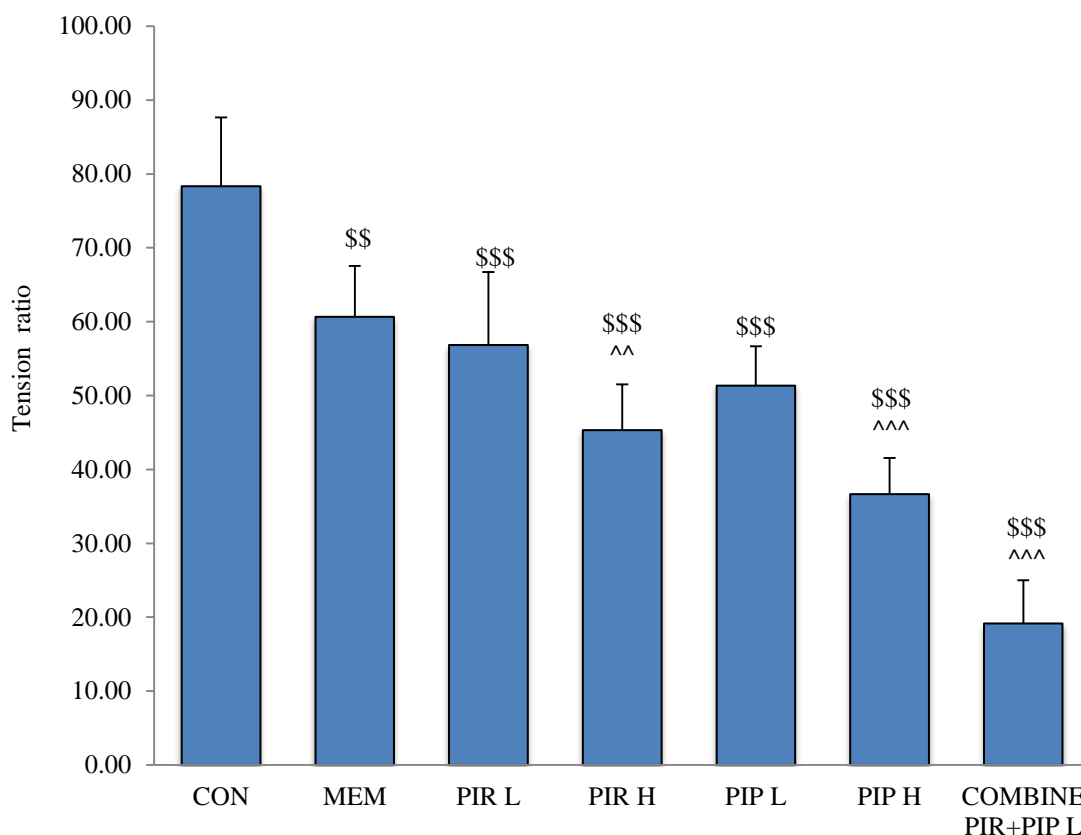


Fig. 40: In vitro Effect of pirfenidone and piperine and its combination:

Data represented as mean \pm SD. CON: Control; PIR: Pirfenidone; PIP: Piperine; MOR: Morphine; NAL: Naloxone; MEM: Memantine; L: low dose; H: high dose. \$\$ $p < 0.01$, \$\$\$ $p < 0.001$ represent significant difference in group 1 vs group 2, 3, 4, 5, 6, 7; ^^ $p < 0.01$, ^^ $p < 0.001$ represent significant difference in division 2 vs group 3, 4, 5, 6, 7 one-way analysis of variance come succed by Tukey's comparison test

5.3. Discussion

In the current study, both the in vivo and in vitro were employed to check the opioid withdrawal syndrome. Both the models of opioid withdrawal have earlier been used by a number of other research groups to validate the role of pirfenidone and piperine involved in opioid withdrawal syndrome (Way *et al.* 1969; Valeri *et al.*, 1995; Capasso and Sorrentino 1997; Munday *et al.*, 1998; Rehni *et al.* 2008a, b; Capasso and Gallo 2009; Rehni and Singh 2011). The two types of animal models

for opioid withdrawal, “acute” (i.e., short period) and “chronic” (i.e., long period), have been useful to understand the concept of opiate tolerance and dependence followed by withdrawal syndrome (De Vries and Shippenberg 2002; Harris and Gewirtz 2005). In acute administration of opioid drug or morphine means the dose of opioid drug is meant for single or multiple dosages over 1 to 2 days only, on the other hand chronic administration of opioid drug or morphine exposure means use of drug atleast minimum of 5 days continuously. (Georges *et al.*, 2000; Laorden *et al.*, 2002). In the present study, chronic administration of morphine has been used for withdrawal syndrome because chronic exposure of opioid drug studies are performed to determine the cellular and molecular mechanism of opioid withdrawal syndrome where as acute exposure of opioid drug may be useful to learn the concept of initial stages of opioid dependence, addiction followed by withdrawal syndrome. (Aston- Jones *et al.*, 1999; Georges *et al.*, 2000; Laorden *et al.*, 2002; Stornetta *et al.*, 1993).

Both the animal models of withdrawal syndrome i.e precipitated and spontaneous were used to understand the study of opioid withdrawal signs. Spontaneous opioid withdrawal happens after the sudden termination or the rapid tapering of chronically administered opioid drug or morphine where as Precipitated opiate withdrawal occurs after the use of an opioid receptor antagonist, which displaces the opioid receptor agonist. In the present investigation, morphine-naloxone group, administration of morphine (5 mg/kg; i.p) twice daily for 5 days (day 1 to day 5) and administration of naloxone (8 mg/kg; i.p) on the day 6 there were significant increase in stereotyped circling, fore paw licking, jumping, withdrawal severity score, rearing behavior, locomotor activity, causes depression, anxiety, analgesia, increased the urination flow, defecation have been seen when taken the comparison of vehicle treated control groups [versus vehicle-control; versus pifenidone and piperine *per se* versus morphine *per se*]. In the precipitated animal model, these withdrawal symptoms were precipitated by administered the dose of naloxone. As we already discussed that naloxone is a competitive μ -opioid receptor antagonists which can be used to precipitate opiate withdrawal symptoms (Reti and Baraban 2003). This drug counter the effects of opiate

overdose, for example heroin or morphine overdose. On the other hand, in spontaneous animal model, morphine was administered for 5 days twice daily, on 6th day the process was terminated and observed the spontaneous response, the withdrawal behavior observed in spontaneous model were less prominent as compared to precipitated animal model.

Animal studies are well suited to test this hypothesis. The symptoms and signs found in humans can be similar in models of compulsive drugs used in mice (Morgan and Christie 2011). It has been approved that animal models have very strong predictive validity that the same opioids will produce dependence in humans beings. These models also have been used to examine a range of adjuncts that can lessen the severity of withdrawal with the goal of facilitating opioid detoxification. For example, animal models have led to the use of the α 2-adrenoceptor agonists clonidine and lofexidine to reduce the severity of opioid withdrawal in humans (Berger and Whistler, 2010). In humans, the withdrawal syndrome consists of signs and symptoms including stomach cramps, diarrhoea, rhinorrhoea, sweating, elevated heart rate and increased blood pressure, irritability, dysphoria, hyperalgesia and insomnia (Cushman and Dole, 1973). In rats and mice, opioid withdrawal signs include jumping, burrowing, circling, rearing, fore paw licking, wet dog shake, fore paw tremor, ptosis, sneezing and straightening. (Laschka *et al.*, 1976; Koob *et al.*, 1992). These signs are readily quantified following administration of antagonists such as naloxone (termed naloxone precipitated withdrawal) or after abrupt cessation of treatment with relatively short acting opioids (termed spontaneous withdrawal)

Various receptors and mechanisms are involved in the pathophysiology of opioid withdrawal. Opiate drugs exert their effects by binding to three opioid receptor types (μ , γ , and κ) and mimicking the actions of endogenous opioid peptides, the endorphins, endomorphins, enkephalins and dynorphins. The μ opioid receptor (MOR) subtype is critical for the rewarding effects of heroin and morphine. The most prominent neuroadaptive changes during morphine induced dependence include desensitization of MORs and upregulation of the cAMP pathway. The mitogen activated protein kinase (MAPK) pathway as well as Ca²⁺ signaling are

also affected during morphine dependence. The primary consequence of morphine withdrawal is superactivation of adenylyl cyclase (AC) and a subsequent overproduction of its downstream signaling molecule, cAMP. Other cAMP actions during withdrawal include PKA-mediated enhanced GABAergic synaptic transmission in brain area such as periaqueductal grey (PAG). This compensatory increase in AC activity in the PAG has been classically associated with opioid dependence development and the withdrawal syndrome (Williams *et al.*, 2001; Bailey and Connor 2005). The brain regions contributing to the physical signs of opiate withdrawal in all the stages include periaqueductal grey (PAG) area, the locus coeruleus (LC), amygdala, ventral tegmental area (VTA), nucleus accumbens (NAcc) (McPhie and Barr, 2009). Among these brain regions, the periaqueductal gray area (PAG) appears to be critical in regulating the complex signs and symptoms of opioid withdrawal (Handong Ouyang *et al.*, 2012).

The toll like receptor has also been implicated in the pathogenesis of opioid withdrawal. These receptors play an important role in morphine withdrawal syndrome in which glial cells, particularly astrocytes, envelop neuronal synapses and participate in the physiological control of synaptic transmission and plasticity via the release of synaptically effective mediators, a process called gliotransmission (Halassa *et al.*, 2007; Haydon *et al.*, 2009). Several studies have reported that glial cells become activated in response to opioids and this glial activation leads to the release of proinflammatory products, including proinflammatory cytokines. In vivo, Hutchinson *et al.* (2009) reported that opioid-induced glial activation has been inferred from (a) morphine-induced upregulation of microglial and astrocytic activation markers (b) morphine-induced upregulation and/or release of proinflammatory cytokines and chemokines (c) enhanced morphine analgesia produced by the glial activation inhibitors fluorocitrate, minocycline or ibudilast (AV411) (d) enhanced morphine analgesia produced by blocking proinflammatory cytokine actions (Raghavendra, *et al.*, 2004; Tawfik, *et al.*, 2005; Cui, *et al.*, 2006; Hutchinson, *et al.*, 2008), and (e) opioid-induced selective activation of microglial p38 MAPK and enhanced morphine analgesia by p38 MAPK inhibitors. In vitro studies also document direct actions of opioids on glial. Morphine binds to an

accessory protein of glial toll-like receptor 4 (TLR4), myeloid differentiation protein 2 (MD-2), thereby inducing TLR4 oligomerization and triggering proinflammatory cytokines (Wang *et al.*, 2012). Direct activation of glial TLR4 induces overexpression of TNF α (Bettoni *et al.*, 2008; Saito *et al.*, 2010). Morphine withdrawal induces astrocytic activation to release TNF α in the PAG (Hao *et al.*, 2011). Thus it may be suggested that increase in level of TNF- α through the activation of glial cells via TLR might play an important role in mediating the progression of opioid dependence and pharmacological modulation of TNF- α might exert an ameliorative effect on opioid withdrawal induced abstinence syndrome in mice (Hao *et al.*, 2011).

Various scientific researches on medicinal herbs and allopathic drugs have reported that these drugs have good antioxidant and anti-inflammatory activities which may provide useful therapeutic interventions against opioid withdrawal syndrome, oxidative stress and overexpression of TNF- α (Hao *et al.*, 2011). It has been suggested by some recent reviews that these drugs have an important role to treat the proinflammatory cytokine in opioid withdrawal. Pirfenidone and piperine have been reported as the important therapeutic interventions for treating various major illnesses diabetes, malignancies, cardiovascular disorders and neurodegenerative diseases (Das *et al.* 2019). The increased level of Tnf- α due to activation of toll like receptor 4 followed by glial cells activation are mainly responsible for the opioid withdrawal syndrome (Ljubuncic *et al.*, 2010; Wang *et al.*, 2014; Moylan *et al.*, 2014). Pirfenidone has widely been studied for its antioxidant and antiinflammatory properties (Poplawski *et al.*, 2010), for inhibiting increased level of oxidative stress and inhibiting the microglial activation and proinflammatory cytokines in certain regions of brain (Limon *et al.*, 2009). This proposed mechanism was further supported by evidence that pre treatment with free radical scavengers reducing the symptoms of morphine withdrawal in mice Abdel-Zaher *et al.*, 2013; Darvishzadeh- Mahani *et al.*, 2012; Abdel-Zaher *et al.*, 2011, Abdel-Zaher *et al.*, 2010). Abdel-Zaher *et al.* (2013) (also reported the possible role of oxidative stress in the development of morphine tolerance and dependence).

In current research, pirfenidone (at both low and high doses), piperine (at both doses) and their combination co-treatment successfully attenuated the opioid withdrawal syndrome in both precipitated and spontaneous animal model with decreased in jumping, circling, fore paw licking rearing withdrawal severity score, locomotor activity, depression, anxiety, analgesic, defecation and urination behaviour significantly. Memantine is a well-known drug use to treat the opioid withdrawal syndrome and was taken as standard drug in the present study. This drug is used for the treatment of opioid withdrawal and are well known for their protective effects against oxidative stress induced by chronic administration of morphine sulphate for 5 days twice daily. (Shankaranarayanan *et al.*, 2013). Pirfenidone at low dose (200 mg/kg; p.o) significantly attenuated the opioid withdrawal syndrome in both the precipitated and spontaneous animal model. but pirfenidone (300 mg/kg; p.o) showed better reversal of opioid withdrawal behavioural alterations Piperine at low dose (10 mg/kg; p.o) significantly attenuated the opioid withdrawal syndrome in both the precipitated and spontaneous animal model. but high dose of piperine (15 mg/kg; p.o) showed better reversal of opioid withdrawal behavioural alterations but the best effect was seen with both pirfenidone and piperine low dose (200 and 15 mg/kg; p.o) combination co-treatment which was significantly better individual drug.

Morphine increased the lipid peroxidation in tissues and led to oxidative DNA damage, protein oxidation and lipid peroxidation in the brain of mice (Ozmen *et al.*, 2007; Qiusheng *et al.*, 2005). In the experiment with mice exposed to morphine there was a decrease in the activity of antioxidant enzymes in the brain such as superoxide dismutase (SOD), catalase, glutathione (GSH) and TBARS leading to reduction in learning ability of the mice (Guzman *et al.*, 2006).

In both the models i.e precipitated and spontaneous, withdrawal syndrome increase in TBARS,SAG levels along increase in GSH and CAT levels were significantly attenuated by pirfenidone (at both low and high doses), piperine (at both doses) and their combination co-treatment. (Pereska *et al.*, 2007). In vitro studies have shown that acute morphine treatment altered the production of various pro-inflammatory cytokines, including TNF- α (Zubelewicz *et al.* 2000). In the

present study, we found that administration of naloxone in morphine-dependent mice increased the level of TNF- α in mice brain, but pirfenidone and piperine could be able to inhibit this cytokines production. In this regard, it has been shown that withdrawal behavior, depression, anxiety, analgesic, locomotor activity were associated with the elevated plasma TNF- α which can be inhibited with combination of both the drugs (pirfenidone and piperine (200 mg/kg and 10 mg/kg) in patients (Li *et al.* 2013; Piletz *et al.* 2009). Then, our data suggested that combination of both the drugs probably could be able to decrease the microglial activation in morphine dependent mice.

In all parameters such as behavioral, biochemical evaluations, both pirfenidone and piperine showed significant attenuating effect at both doses but best effect was seen with pirfenidone+piperine combination co-treatment that was significantly higher than the standard drug.

In the overall study, pirfenidone, piperine and their combination showed better effect in midbrain region. The exact reason responsible for the effectiveness of pirfenidone and piperine in the present study is not known but it can be speculated that pirfenidone and piperine might have shown its efficacy due to its reported activities such as antioxidant, anti-inflammatory properties (Poplawski *et al.*, 2010), pro-inflammatory cytokines inhibition and microglial activation in hippocampus (Limon *et al.*, 2009) attenuating inflammatory cytokines, TNF- α mediated by microglial cells through causing reduction in activation of TLR (Kim *et al.*, 2015). Additionally, the inhibitors of NMDA receptors, such as conidine, memantine have been approved clinically for treating opioid withdrawal syndrome. All above discussed reported activities of pirfenidone+piperine suggest its possible efficacy in opioid withdrawal syndrome. However, further studies are needed to explore the mechanism of synergism among these test substances which could add more value to the present study.

It has been reported that pirfenidone, a pyridine molecule, possesses anti-inflammatory, anti-oxidant and antifibrotic properties. Previous studies have shown that pirfenidone and piperine significantly and dose dependently reduced the

formation of intracellular reactive oxygen species (ROS) and tumour necrosis factor alpha (TNF- α) in various diseases (Lasky 2002). In the present investigation, the administration of pirfenidone and piperine, during morphine treatment protocol, dose- dependently attenuated the naloxone precipitated opioid withdrawal and spontaneous opioid withdrawal syndrome. Therefore, it might be postulated that this decrease in the intensity of the naloxone precipitated withdrawal syndrome and spontaneous opioid withdrawal syndrome, oxidative stress and TNF- α by pirfenidone and piperine may be due to reduced level of TNF- α which is involved both the models of opioid withdrawal syndrome via TLR 4 and glial cells activation induced by chronic morphine administration

The exact mechanism lying behind the effectiveness of piperine in the present study is not clear but based on present results and previous reports, it can be speculated that the effectiveness of piperine in present study might be due to its reported activities such as antioxidant, anti-inflammatory effects, inhibition of proinflammation and neuroprotective effects (Jin *et al.*, 2013; Akhavan and Ghasemi, 2016; Zha *et al.*, 2018). An increase in TNF- α and oxidative stress causes the withdrawal syndrome has also been shown in the neurons of the brains of human as well as rodents. However further studies are needed to establish the exact mechanism of pirfenidone in opioid withdrawal syndrome.

6. SUMMARY AND CONCLUSION

6.1. Summary

The present study was aimed to study the effect of modulation of tumor necrosis factor α by pirfenidone and piperine on opioid withdrawal syndrome. Withdrawal syndrome was precipitated by administration of morphine (5 mg/kg, i.p) twice daily for a period of 5 days (day 1 to day 5) followed by an injection of naloxone (8 mg/kg, i.p) on day 6, 2 hr after morphine injection. In the second animal model, spontaneous opioid withdrawal, administration of morphine (5 mg/kg, i.p) twice daily for a period of 5 days (day 1 to day 5) and after that discontinue the morphine, observed the spontaneous behavioral parameters. In vehicle control groups, the animals were administered with vehicle of morphine and pirfenidone low dose (200 mg/kg; p.o) high dose (300 mg/kg; p.o) and piperine low dose (10 mg/kg; p.o) high dose (15 mg/kg; p.o) and pirfenidone + piperine (200 mg/kg; p.o and 10 mg/kg; p.o) combination of both the drugs from day 1 to day 5 and vehicle of naloxone on day 6, 2 hr after administration vehicle of morphine and discontinue the morphine on 6th day in precipitated and spontaneous animal model respectively.

Behavioral observations were made in two phases immediately on 6th day in both models of opioid withdrawal syndrome. The observations were made in a transparent perspex observation chamber. The behavioral parameters which were observed in the present study are jumping frequency, withdrawal severity score, rearing frequency, fore paw licking and circling frequency, locomotor activity, depressant activity, anxiety, urination frequency, defecation frequency. Two observers simultaneously observed each mice for all the withdrawal measures, and the mean value of both observations was recorded. Initial 30-min observation period immediately after naloxone administration was the segment of observation period that was used to assess withdrawal severity score and jumping frequency in mice, while the second 30 min observation period represented the segment of observation period that was used to assess the frequency of rearing, fore paw licking and circling, locomotor activity, depressant activity, anxiety, urination frequency, defecation frequency.

Both the precipitated and spontaneous withdrawal models were used to study opiate withdrawal signs. Spontaneous opiate withdrawal occurs after the sudden discontinuation or the rapid tapering of chronically administered opiates. Precipitated opiate withdrawal occurs after the use of an opioid receptor antagonist, which displaces the opioid receptor agonist. In the present investigation, morphine-naloxone group, administration of morphine twice daily for 5 days (day 1 to day 5) and administration of naloxone on the day 6 there were significant increase in stereotyped jumping, circling, fore paw licking, rearing and withdrawal severity score behavior, locomotor activity, causes depression, anxiety, analgesia, increased the urination flow, defecation have been seen when compared to that of vehicle treated control groups [versus vehicle-control; versus pirfenidone and piperine *per se* versus morphine *per se*]. These withdrawal symptoms were precipitated by administration of naloxone. Naloxone is the most common competitive μ -opioid receptor antagonists used to precipitate opiate withdrawal. It is used to counter the effects of opiate overdose, for example heroin or morphine overdose. It has an extremely high affinity for μ -opioid receptors in the central nervous system and causes rapid blockade of opioid receptors which often produces rapid onset of withdrawal symptoms. Similarly in spontaneous animal model, morphine was administrated for 5 days twice daily, on 6th day the process was terminated and observed the spontaneous response, the withdrawal behavior observed in spontaneous model were less prominent as compared to precipitated animal

It is clearly indicated by the present results that pirfenidone and piperine maybe modulate the increased level of TNF- α suggested for their possible therapeutic effect in opioid withdrawal syndrome and their combination may be explored further for its synergistic effect in treatment of opioid withdrawal syndrome

6.2 Conclusion

The present study concluded that both pirfenidone and piperine attenuated the behavioural, biochemical, alterations due to increased oxidative stress, increased level of TNF- α with effects seen with pirfenidone and piperine and best effect with pifenidone + piperine combination. These effects of pirfenidone and piperine can

be interpreted in light of their ability to produce reduction in oxidative stress and overexpression of TNF- α in the midbrain of mice. Pirfenidone + piperine combination showed maximum ameliorative effect in all parameters. So it can be suggested to further explore the pirfenidone + piperine combination for its beneficial effect in the opioid withdrawal syndrome. However, the exact mechanism of these drugs at molecular level should be further explored to warrant its ameliorative effect in opioid withdrawal syndrome.

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41500067

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Rani Ka Bagh, Amritsar
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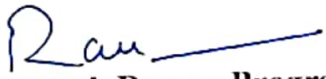
Dear Student,

Subject: Letter of Candidacy for Ph.D.

We are pleased to inform you that the Department Doctoral Board has approved your candidacy for the Ph.D. Programme on 1st October 2016 by accepting your research proposal entitled: "MODULATION OF TNF-ALPHA BY PIRFENIDONE AND PIPERINE IN OPIOID WITHDRAWAL SYNDROME" under the supervision of Dr. Navneet Khurana..

As a Ph.D candidate, you are required to abide by the conditions, rules and regulations laid down for Ph.D. Programme of the University, and amendments, if any, made from time to time.

We wish you the very best and in case you have any query related to your Programme, please do not hesitate to contact School of Research Degree Programmes.


HOS (Research Degree Programmes)



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Provisional Academic Transcript

Student Name : Gurpreet Bawa Programme : PSD0-TN2 :: Doctor of Philosophy in Pharmacology
 Registration no. : 41500067
 Father's Name : Mr. Yashpal Singh Bawa Mode : Parttime Date of initial registration : August,2015
 Mother's Name : Ms. Narinderjit Bawa

TERM-I		TGPA : 9.71	Equivalent percentage : 87.39 %	
S.No.	Course		Credits	Grade
1	GEN708 :: RESEARCH METHODOLOGY		4	A+
2	GEN709 :: WORKSHOP ON PROJECT WRITING		1	A+
3	GEN710 :: WORKSHOP ON DATA ANALYSIS		2	A

<p>This is a provisional transcript and is valid till a regular transcript is issued Student yet not completed the programme.</p>	<p>CGPA : 9.71 Equivalent percentage : 87.39 %</p>
--	---

[Signature]
 Dy. Dean Examination
 Lovely Professional University
 Phagwara (Punjab)

CGPA & Grades
 (Officer in Charge)
 Checked & Verified by

Print Date: 06-09-2016
 Place : Phagwara (Punjab)

LOVELY PROFESSIONAL UNIVERSITY

BASIS OF EVALUATION AND GRADING

1. University uses Broadband Letter Grades to report a student's performance. Each letter grade indicates the level of performance in a course and has an associated grade point value as per the following table:

Letter Grade	Performance	Grade Points
A+	Outstanding	10
A	Very Good	9
B	Good	8
B-	Above Average	7
C	Average	6
C-	Below Average	5
D	Marginal Pass	4
E	Reappear	0
F	Fail	0
I	Incomplete	-
R	Resubmission	-
S	Satisfactory (for zero credit courses only)	-
U	Unsatisfactory (for zero credit course only)	-

2. A+, A, B, B-, C, C- and D are Pass grades. E is a temporary grade indicating permission to reappear in the re-appear examination to improve it to a passing grade, and F is a fail Grade. I represents an incomplete result and is replaced with the grade that student obtains after completing the requirements. R represents resubmission in case of projects/dissertations or other similar courses. S and U grades are awarded only in case of courses with zero credit: S for satisfactory performance and U for failing in a course.

3. A student's overall academic performance within a given term is represented by Term Grade Point Average (TGPA), and the overall performance in all the courses completed up to and including the current term is represented by Cumulative Grade Point Average (CGPA). TGPA and CGPA are calculated as the weighted averages of the grades:

$TGPA / CGPA = \frac{\sum C_i G_i}{\sum C_i}$, where C represents the credits associated with a course and G represents the grade points of the letter grade in that course.

4. If a conversion to marks is required, the nominal percentage of marks may be calculated by multiplying the CGPA with 9.0
5. The CGPA requirement for the award of degree / diploma / certificate will be 5.0 or above for Regular Programmes and 4.0 or above for Distance Education Programmes, subject to getting a passing grade in each of the courses individually, and satisfying other conditions as specified in the examination ordinances / rules and programme details.

6.

Division	CGPA
First with distinction	9.0 or more subject to the conditions that student has passed all the courses in the 1st attempt.
First	7.0 or more but less than 9.0

7. This academic transcript can be authenticated by submitting the certificate number to www.lpu.in/authenticate or by writing to Examination Division, Lovely Professional University, Jalandhar - Delhi GT Road, Phagwara 144806, Punjab, India.

International Conference of Pharmacy [ICP - 2017]



Society of
Pharmaceutical Education
& Research (SPER)



LOVELY
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Theme : "Pharmacists: Catalysts for Change"

April 7-8, 2017

Venue : School of Pharmaceutical Sciences, Lovely Professional University, Phagwara [Punjab] India

Certificate

This is to certify that Prof./Dr./Mr./Mrs./Ms. Gurpreet Bawa

has successfully participated in the International Conference of Pharmacy [ICP - 2017]

as delegate

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President,
Society of Pharmaceutical Education
& Research, Gwalior [M.P.] India

Dr. Upendra Nagaich
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Prof./Dr./Mr./Ms. Gurpreet Bawa

from Department of Pharmaceutical Sciences, Lovely Professional University

participated in GHG PPU sponsored conference & presented a poster entitled

Pathways Involved In Opioid Withdrawal Syndrome during

National Conference on "Challenges and Future Vision in Phytomedicines - An Alternative to Modern Medicine"

organized by GHG Khalsa College of Pharmacy, Gurur Sadhar, Ludhiana, Punjab

in collaboration with APP Punjab State Branch on 22nd day of September 2017.

Dr. Satvinder Kaur
Convener & Principal
GHG Khalsa College of Pharmacy
Gurur Sadhar, Ludhiana, Punjab

Dr. Rajiv Dahiya
Organizing Chairman & President APP
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A Review on the viral infection caused with SARS-CoV2 and management by medications that are utilized in the therapeutics of COVID-19

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Submitted: 01-12-2021

Revised: 11-12-2021

Accepted: 14-12-2021

ABSTRACT: COVID infection is one of the deadly viral infections across the globe that is generally etiolate by the SARS-CoV-2 infection and spread through the transmission and by the infected person. This infection originated from the

I. INTRODUCTION

COVID infection 2019 is a pandemic viral issue; began in Wuhan, China, in December 2019



CLINICAL USES OF PIPERINE: A REVIEW

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Article Received on
22 July 2021,

Revised on 12 August 2021,
Accepted on 02 Sept. 2021

DOI: 10.20959/wjpr202111-21615

ABSTRACT

Main Substance of piper nigrum is Piperine having pungency that is accountable for hundreds of clinical dietary utility and worldwide popularity as a food ingredient. It is act as a edible ingredients and utilized as a edible preservations with the application, that is utilized in the conventionally drug for various demonstrations, that assured in

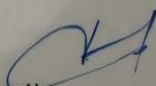
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[Under the Aegis of Lovely Professional University, Jalandhar-Delhi G.T. Road, Phagwara (Punjab)]


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Certificate of Participation

This is to certify that **Ms. Gurpreet Bawa** D/o Sh. Yashpal Singh Bawa participated in **Hands on Training to Improve Quality of Research Conducted on Experimental Animals** organized by Human Resource Development Center, Lovely Professional University from **March 23, 2018 to March 24, 2018** and obtained 'O' Grade.


Prepared by
(Administrative Officer -Records)


Head
Human Resource Development Center


Head
Division of Human Resource

Date of Issue: 24-03-2018
Place: Phagwara (Punjab)



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QJG Quantum Jump Consulting Group

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This is to certify that Mr./Ms. GURPREET BAWA
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Food & Drugs Administration, Punjab,
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To

**M/s Ms. Gurpreet Bawa (Research Scholar),,
School of Pharmaceutical Sciences, Lovely
Professional University, Phagwara,
Distt. Kapurthala.**

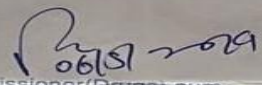
No. Drugs (10) Pb. 2019/ 2165
Dated 7-5-19

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Reference your application received Dated Nil in this office through Drugs Inspector, Kapurthala letter no. Drugs/2019/101 dated 20.3.2019 on the subject noted above.

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Dated

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11
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