# TREATMENT OF DEPRESSION IN RATS EXPOSED TO CHRONIC UNPREDICTABLE MILD STRESS USING SYNTHETIC DERIVATIVES OF ISATIN

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**THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY**
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In partial fulfillment of the requirements for the award of Degree of
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**IN**
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Submitted by
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**COIMBATORE – 641 044**


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# CERTIFICATE

This is to certify that the M. Pharm., dissertation entitled **"TREATMENT OF DEPRESSION IN RATS EXPOSED TO CHRONIC UNPREDICTABLE MILD STRESS USING SYNTHETIC DERIVATIVES OF ISATIN"** being submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment of **Master of Pharmacy** programme in **Pharmacology**, carried out by **Ajin P Kurian (Register No: 261825101)** in the Department of Pharmacology, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, under my direct supervision and guidance to my full satisfaction.

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# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| MDD | - | Major Depressive Disorder |
| WHO | - | World Health Organization |
| HPA | - | Hypothalamus-Pituitary-Adrenal |
| HPT | - | Hypothalamus-Thyroid-Adrenal |
| PFC | - | Prefrontal Cortex |
| ACC | - | Anterior Cingulate Cortex |
| VMPFC | - | Ventromedial Prefrontal Cortex |
| DLPFC | - | Dorsolateral Prefrontal Cortex |
| NE | - | Norepinephrine |
| DA | - | Dopamine |
| 5-HT | - | Serotonin |
| DOPA | - | 3, 4-Dihydroxy Phenylalanine |
| MAO- A | - | Monoamine Oxidase A |
| MAO- B | - | Monoamine Oxidase B |
| COMT | - | Catechol O-Methyl Transferase |
| PNMT | - | Phenylethanolamine N-Methyltransferase |
| GABA | - | Gaba Amino Butyric Acid |
| PTSD | - | Post-Traumatic Stress Disorder |
| CRH | - | Corticotrophin-Releasing Hormone |
| TNF-A | - | Tumour Necrosis Factor-A |
| ACTH | - | Adreocorticotropic Releasing Hormone |
| GR | - | Glucocorticoid Receptor |
| BDNF | - | Brain-Derived Neurotrophic Factor |

| | | |
|---|---|---|
| GAD | - | Glutamate Decarboxylase |
| BBB | - | Blood- Brain Barrier |
| CNS | - | Central Nervous System |
| CSF | - | Cerebrospinal Fluid |
| FAD | - | Flavin Adenosine Dinucleotide |
| IDO 1 | - | Indoleamine 2,3-Dioxygenase |
| TDO | - | Tryptophan 2,3-Dioxygenase |
| NMDA | - | N-Methyl-D-Aspartate |
| SDI | - | Synthetic Derivative of Isatin |
| CADD | - | Computer Aided Drug Design |
| GPCR | - | G - Protein Coupled Receptors |
| PDB | - | Protein Data Bank |
| HBD | - | Hydrogen Bond Donors |
| HBA | - | Hydrogen Bond Acceptors |
| SMILES | - | Simplified Molecular Input Line Entry System |
| BSA | - | Bovine Serum Albumin |
| Ro5 | - | Rule of Five |
| KI | - | Inhibitory Constant |
| UV | - | Ultra Violet |
| IR | - | Infra-Red |
| NMR | - | Nuclear Magnetic Resonance |
| SPT | - | Sucrose Preference Test |
| TST | - | Tail Suspension Test |
| FST | - | Forced Swim Test |
| EPM | - | Elevated Plus Maze |
| Gpx | - | Glutathione Peroxidase |
| GSH | - | Reduced Glutathione |
| TRP | - | Tryptophan |
| KYN | - | Kynurenine |
| CMC | - | Carboxy Methyl Cellulose |

| | | |
|---|---|---|
| CAT | - | Catalase |
| SOD | - | Superoxide Dismutase |
| MDA | - | Malondialdehyde |
| GSSH | - | Glutathione Reductase |
| TCA | - | Trichloro Acetic Acid |
| ANOVA | - | Analysis of Variance |
| SEM | - | Standard Error Mean |

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## INTRODUCTION

Among all the diseases and disorders, the most complex and disturbing of all age groups are the psychological and neurological disorders. Psychiatric disorders have proved to be a major health problem in the recent years. These disorders not only influence the individual's health and mental status but also its social, socioeconomic and family life. [1]

Mental disorders comprise a broad range of problems, with different symptoms. However, they are generally characterized by some combination of abnormal thoughts, emotions, behaviour and relationships with others. Examples are schizophrenia, depression, intellectual disabilities and disorders due to drug abuse. [1] Most of these disorders can be successfully treated. The burden of mental disorders continues to grow with significant impacts on health and major social, human rights and economic consequences in all countries of the world. [2]

## EPIDEMIOLOGY

Depression is a common mental disorder and one of the main causes of disability worldwide. Globally, an estimated 300 million people are affected by depression. More women are affected than men. Depression is characterized by sadness, loss of interest or pleasure, feelings of guilt or low self-worth, disturbed sleep or appetite, tiredness, and poor concentration. People with depression may also have multiple physical complaints with no apparent physical cause. Depression can be long-lasting or recurrent, substantially impairing people's ability to function at work or school and to cope with daily life. At its most severe, it can lead to suicide. [2]

Thoughts of guilt, worthlessness, and suicide are common. Coronary artery disease, diabetes, and stroke appear to be more common in depressed patients, and depression may considerably worsen the prognosis for patients with a variety of comorbid medical conditions. Recently conducted world mental health surveys indicate that major depression is experienced by 10-15% people in their lifetime and about 5% suffer from major depression in any given year. Lifetime prevalence of all depressive disorders taken together is over 20%, that is one in five indiviuals. [1]

Depression is often underdiagnosed and undertreated. This is of particular concern due to the inherent risk of suicide associated with depression. Approximately 10-15% of those with

severe depression attempt suicide at some time. Thus, it is important that symptoms of depression be recognized and treated in a timely manner. [2]

**TYPES OF DEPRESSION**

Depression, in general, is classified as major depression (i.e., unipolar depression) or bipolar depression (i.e., manic depressive illness). Lifetime risk of unipolar depression is ~15%. Females are affected twice as frequently as males. Depressive episodes are characterized by depressed or sad mood, pessimistic worry, diminished interest in normal activities, mental slowing and poor concentration, insomnia or increased sleep, significant weight loss or gain due to altered eating and activity patterns, psychomotor agitation or retardation, feelings of guilt and worthlessness, decreased energy and libido, and suicidal ideation, occurring most days for a period of at least 2 weeks. In some cases, the primary complaint of patients involves somatic pain or other physical symptoms and can present a diagnostic challenge for primary care physicians. Depressive symptoms also can occur secondary to other illnesses such as hypothyroidism, Parkinson's disease, and inflammatory conditions. [3]

There are two distinct types of depressive syndrome, namely unipolar depression, in which the mood swings are always in the same direction, and bipolar affective disorder, in which depression alternates with mania. Mania is in most respects exactly the opposite, with excessive exuberance, enthusiasm and self-confidence, accompanied by impulsive actions, these signs often being combined with irritability, impatience and aggression. [4]

Unipolar depression is commonly (about 75% of cases) non-familial, clearly associated with stressful life events, and accompanied by symptoms of anxiety and agitation; this type is sometimes termed reactive depression. Other cases (about 25%, sometimes termed endogenous depression) show a familial pattern, unrelated to external stresses, and with a somewhat different symptomatology. [4]

Bipolar depression, once called manic depression, is characterized by moods that shift from severe highs (mania) or mild highs (hypomania) to severe lows (depression). The mood episodes associated with the disorder persist from days to weeks or longer and may be dramatic. Severe changes in behavior go along with mood changes. [5]

This usually appears in early adult life, is less common and results in oscillating depression and mania over a period of a few weeks. There is a strong hereditary tendency, but

no specific susceptibility genes have been identified either by genetic linkage studies of affected families, or by comparison of affected and non-affected individuals. [5]

**ETIOLOGY**

Like most psychiatric disorder, the cause of affective disorder remains unknown in depression. Following factors which make the person susceptible to depression include, biochemical, endocrine, genetic, environmental and hormonal. [6]

**Genetic factor**

Serotonin transporter gene is a risk factor for depression. Many common disorders which are also influenced by genes include high blood pressure and diabetes. Cystic fibrosis and Huntington's disease may be caused due to the single defective gene. [6]

**Biochemical factor**

Depression is associated with deficiency of neurotransmitter in a certain region of the brain includes dopamine, norepinephrine, and 5-hydroxytryptamine. Antidepressants increase the level of this neurotransmitter in the brain. [6]

**Endocrine factor**

Over activity of hypothalamus-pituitary-adrenal (HPA) axis is associated with the affective disorder. Cushing's syndrome and hypothyroidism are connected with HPA and hypothalamus-thyroid-adrenal (HPT) axis dysfunction. [7]

**Environmental factor**

Environmental factors responsible for depression such as water, air, synthetic chemicals, food additives and food pollution, hormones, pesticides, drugs and industrial by-products are bombarding our bodies at an extreme rate. Other sources include stress electrical pollution, natural disasters, noise pollution and other catastrophic environmental events. Some events include the death of a loved one, divorce, job loss, financial problems and disabling illness or injury sometimes called as social and relational causes of depression. [7]

**RISK FACTORS OF DEPRESSION**

Numerous studies have investigated the psycho-social environmental and biological risk factor associated with the development of depressive illness. Epidemiological research is the key to the identification of risk factors for mental disorders. Familiarity with the risk factors for major depressive disorders may help to recognize or diagnose this common and serious psychiatric illness. [8]

1. History of prior episodes of depression
2. Family history of depressive disorders especially in first-degree relatives.
3. History of suicide attempts
4. Female gender
5. Age of onset - before age 40
6. Postpartum period
7. Co-morbid medical illness
8. Absence of social support.
9. Negative, stressful life events
10. Active alcohol or substance abuse.
11. Unmarried, Divorced or Separated status

**THE NEUROANATOMY OF DEPRESSION**

The region in the brain associated with depression include hippocampus, basal ganglia and amygdala and cortical brain regions. Abnormalities in the structure and function of these regions in patient's exhibit distinct pathological changes in the brain and have been found to be associated with depression. [8,9]

**Prefrontal cortex (PFC)**

The PFC lies anteriorly to the premotor (involved in the planning of complex motor actions) and the primary motor area (mediates conscious movement) of the frontal cortex and has 'heteromodal' function, integrating complex sensorimotor information with motivation and affect. It is divided into three major sections:

- The dorsolateral,
- The paralimbic (orbital and medial aspects of PFC)
- The anterior cingulate cortex (ACC).

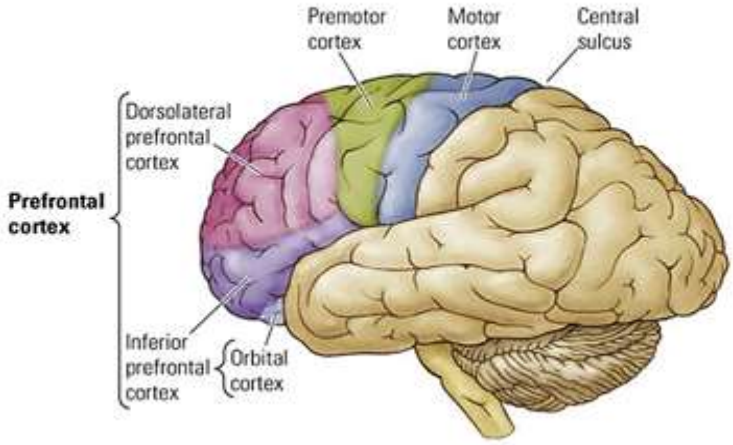



**Fig.1: Position of Prefrontal cortex**

The ventromedial prefrontal cortex (VMPFC) and the dorsolateral prefrontal cortex (DLPFC) connect with each other via the cingulate gyrus and the hippocampus. The VMPFC is necessary for the normal generation of emotions, in particular social emotions and it regulates autonomic and neuroendocrine responses, pain modulation, aggression and sexual and eating behaviours. The orbital PFC plays a role in correcting behavioural or emotional responses (generated in part by the amygdala). [9,10]

The DLPFC has been implicated in cognitive control, solving complex tasks, maintenance and manipulation of information in working memory. The PFC, the amygdala and particularly the hippocampus are the brain structures most widely studied in relation to depression. The VMPFC show increased activity while the DLPFC shows a decrease in activity, in depression. Decreased activity of the DLPFC in depression has been associated with psychomotor retardation and anhedonia. Response to treatment is associated with a decrease in metabolic activity, and chronic antidepressant drug treatment reduces metabolism in the amygdala and ACC in subjects with persistent, positive treatment response. [10]

**Amygdala**

The amygdala is involved in recruiting and coordinating cortical arousal and neuroendocrine response to underdetermined (surprising and ambiguous) stimuli as well as in emotional learning and memory. Abnormal activation of the amygdala correlates with the severity of the depression. [11] They have been implicated in the tendency to ruminate and may

also play a role in bipolar depression and anxiety. The evidence in terms of the amygdala volume in depression has been inconsistent. A recent meta-analysis of MRI studies, which took into account the possible role of medication on the size of the amygdala, demonstrated that this is actually reduced in unmedicated depressed patients. [11,12]

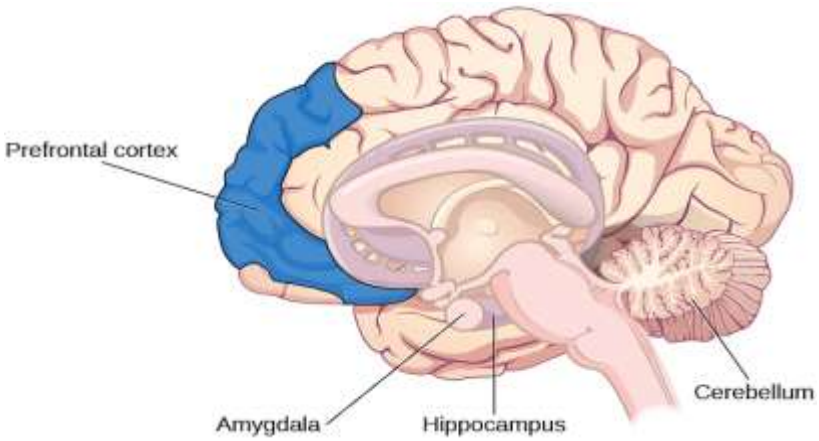



**Fig. 2: Position of Amygdala and Hippocampus**

**Hippocampus**

The hippocampus is the most widely studied brain structure in relation to depression, both in animals and man for several reasons:

i. It plays a fundamental role in learning and memory; dysfunction in the hippocampus may be responsible for inappropriate context-dependent emotional responses;

ii. It is rich in corticosteroid receptors and is closely linked anatomically and physiologically to the hypothalamus via a bundle of axons, the fornix, providing regulatory (inhibitory) feedback to the HPA axis;

iii. It is one of the two brain areas where neo-neurogenesis is known to continue in the mature brain in animals and man, hence its high capacity for neuroplasticity. [12, 13]

Although hippocampal function may be affected, as shown by impairment in hippocampus-dependent verbal memory tests, both in patients with first-episode depression and those with multiple episodes, it is only in the latter group that the hippocampal volume is reduced; this would suggest that dysfunction of the hippocampus predates detectable structural changes. [13]

The system also links with relevant structures in the midbrain/brainstem (for example,

the serotonergic raphe nuclei and the adrenergic nucleus coeruleus). The cortical- striato-pallido-thalamic-limbic circuit is responsible for maintaining emotional stability and appropriate responses to emotional stimuli as well as regulating neurotransmission, autonomic and neuroendocrine function. The dysregulation may be responsible for the clinical depressive syndrome and the associated autonomic, neuroendocrine disturbances and other visceral functions. [14]

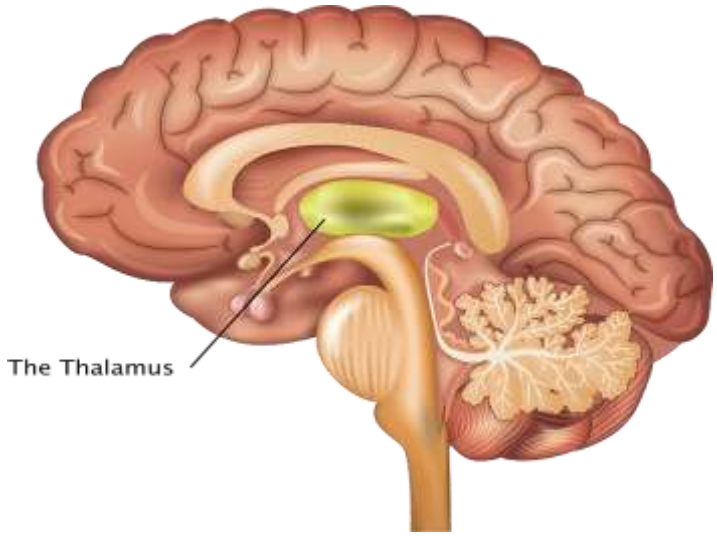



**Fig. 3: Position of the Thalamus**

**Thalamus**

It is a midline symmetrical structure of two halves, within the vertebrate brain, situated between the cerebral cortex and the midbrain. The thalamus is involved in the transmission of sensory information and regulation of learning speech, behavioural reactions, movement, and thinking. The thalamus plays a major role in regulating arousal, the level of awareness, and activity. Damage to the thalamus can lead to bipolar depression as well as permanent coma. [13,14]

**PATHOPHYSIOLOGY OF DEPRESSION**

**Monoamine hypothesis**

The monoamine hypothesis of depression suggests that depression is related to a deficiency in the amount or function of cortical and limbic serotonin (5-HT), norepinephrine (NE), and dopamine (DA).

1. Noradrenergic pathway in the CNS
2. Serotonergic pathway in the CNS
3. Dopaminergic pathway in the CNS

In monoamine systems, reuptake of the transmitter is the main mechanism by which neurotransmission is terminated; thus, inhibition of reuptake can enhance neurotransmission, presumably by slowing clearance of the transmitter from the synapse and prolonging the time of the transmitter in the synapse. Enhancing neurotransmission may subsequently lead to adaptive changes. Reuptake inhibitors inhibit the neuronal serotonin (5-hydroxytryptamine; 5-HT) transporter, the neuronal norepinephrine (NE) transporter; or both. Similarly, the first-generation drugs, which include monoamine oxidase inhibitors (MAOIs) and tricyclic antidepressants (TCAs), also enhance monoaminergic neurotransmission: the MAOIs by inhibiting monoamine metabolism and thereby enhancing neurotransmitter storage in secretory granules, the TCAs by inhibiting 5-HT and norepinephrine reuptake. [15]

**Neuroendocrine hypothesis**

In depression, hypothalamic-pituitary-adrenal axis (HPA axis) and hypothalamic-pituitary thyroid- axis (HPT axis) play an important role. The hyperactivity of HPA axis and defective HPA-axis-glucocorticoid feedback mechanisms are widely reported in the neurobiology of depression. [16]

Depression is known to be associated with a number of hormonal abnormalities. Among the most replicated of these findings are abnormalities in the HPA axis in patients with major depressive disorder (MDD). Moreover, MDD is associated with elevated cortisol levels, non-suppression of adrenocorticotropic hormone (ACTH) release in the dexamethasone suppression test, and chronically elevated levels of corticotropin-releasing hormone (CRH). [4]

CRH concentrations in the brain and cerebrospinal fluid of depressed patients are increased. Therefore, CRH hyperfunction, as well as monoamine hypofunction, may be associated with depression. [16]

Thyroid dysregulation has also been reported in depressed patients. Up to 25% of depressed patients are reported to have abnormal thyroid function. These include a blunting of response of thyrotropin to thyrotropin-releasing hormone, and elevations in circulating thyroxine during depressed states. [16]

**Neuroinflammatory hypothesis**

The inflammatory hypothesis of depression proposes the association of inflammation with diminished neurogenesis and increased neurodegeneration in the brain of MDD patients. Cytokines such as tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1) and IL-6, and downstream acute-phase reactants more notably C-reactive protein (CRP) are among the most reported increased inflammatory biomarkers in peripheral blood in depression. In fact, stress-

induced activated inflammatory responses within the CNS may lead to oxidative stress and neuro-progressive processes in major depression. In animal models exposed to either acute or chronic stress, the excessive production of cytokines can cause diminished neurotrophic support and neurogenesis, features that are enhanced by appropriate cytokine response within the physiological range. Of note, neuroinflammation can enhance the oxidative status in the CNS, stimulating the production of nitric oxide, another characteristic observed in the pathophysiology of depression. [16,17]

Astrocytes and microglia as well as neurons can synthesize and secrete cytokines under stress-related pathophysiological conditions. Alteration in their functional properties and abnormalities of these brain cells have been reported in suicide victims with depression and in depressed patients, where neuroimaging studies revealed enhanced activation of microglial cells and astrocytes. When these brain cells are altered, they may further attract activated immune cells from the periphery. Cytokines influence the neuroendocrine function by having an impact on the HPA axis activity. The effect has been reported to be through stimulation of CRH, ACTH, and cortisol expression and release. Pro-inflammatory cytokines may generate glucocorticoid resistance by directly affecting the functional capacity of the GR. They may reduce GR ligand and DNA binding abilities, inhibit GR translocation to the nucleus, and influence GR protein-protein interactions. cytokines activate the mitogen-activated protein kinase-signalling pathway in the cytoplasm leading to phosphorylation of the receptor protein, thus diminishing GR transcriptional activity. [17]

**Neurotrophic and neuroplasticity hypothesis**

Neurotrophin constitute a family of 4 distinct secreted growth factors (nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) which upon binding to membrane receptors in the target neurons activate an intracellular cascade which promotes survival and trophic effects. Each neurotrophin binds with high affinity to specific members of the tyrosine kinase receptor family (Trk receptors; NGF binds to TrkA, BDNF and NT-4 bind to TrkB and, NT-3 binds not only to TrkC but also to the other Trk receptors with low affinity). In addition, all the neurotrophins bind with low affinity to p75 (NTR), which not only is a very different receptor, responsible for storing and transporting neurotrophins, but also promotes neuronal cell death to sculpt neuronal circuits during development. [17]

**Brain-derived neurotrophic factor**

There is substantial evidence that nerve growth factors such as brain-derived neurotrophic factor (BDNF) are critical in the regulation of neural plasticity, resilience, and neurogenesis. The evidence suggests that depression is associated with the loss of neurotrophic support and that effective antidepressant therapies increase neurogenesis and synaptic connectivity in cortical areas such as the hippocampus. BDNF is thought to exert its influence on neuronal survival and growth effects by activating the tyrosine kinase receptor B (TrK-B) in both neurons and glia. Animal and human studies indicate that stress and pain are associated with a drop in BDNF levels and that this loss of neurotrophic support contributes to atrophic structural changes in the hippocampus and perhaps other areas such as the medial frontal cortex and anterior cingulate. The hippocampus is known to be important both in contextual memory and regulation of the hypothalamic-pituitary-adrenal (HPA) axis. [16]

**Cholinergic hypothesis**

The cholinergic system is responsible for a number of CNS functions including arousal, attention, learning, and memory. The depression is often associated with cognitive impairments, mood disturbances and other autonomic, endocrine and sleep-wake abnormalities. The cholinergic neurons are generally antagonistic to catecholaminergic neurons and led to the cholinergic-noradrenergic imbalance. This suggested that an imbalance between these systems resulted in depression. The acetylcholine (ACh) significantly mediates the neuroendocrine and physiological responses to stress and facilitates the release of several stress-sensitive neurohormones and peptides including corticotrophin releasing factor (CRF), ACTH. The antidepressant may reduce the symptom of depression through blockade of nAChRs involved in stress-induced activation of HPA axis. [17]

**MONOAMINE OXIDASE**

The discovery of the first MAO, originally known as tyramine oxidase, has paved the way for researchers to explore the potential of MAOs as biological targets and therapeutic development, mainly related to neurological diseases. In the central and peripheral nervous system (CNS and PNS), MAOs play an important role by modulating the levels of monoamine neurotransmitters. [16]

MAOs are flavin adenine dinucleotide (FAD) co-factor-dependent enzymes localized on the mitochondrial outer membrane that catalyse the oxidation of endogenous and xenobiotic monoamines including dopamine, serotonin and epinephrine in the CNS (Fig.4-A). The two isoforms of MAO are designated as MAO-A and MAO-B, which are recognized by their

distinct substrate and inhibitor selectivity. MAO-B preferentially catalyses the oxidation of benzylamine and phenylethylamine and is inhibited by selegiline, whereas MAO-A preferentially catalyses the oxidation of serotonin and norepinephrine and is inhibited by clorgyline. Inhibition of MAO-A and MAO-B enzymes significantly elevates the level of vital neurotransmitters which is essential for the clinical manifestation of the patients with MDD. [] Catecholamines such as dopamine (DA), adrenaline (epinephrine), noradrenaline (norepinephrine, NE), tryptamine, and tyramine are substrates for both MAO isoforms (Fig. 4-B). [16,17]

However, DA is mainly metabolized by MAO-B in *substantia nigra*, where MAO-B is the main distributed isoform in glial cells and the increased MAO-B activity is associated with loss of DA in the human brain. [16]

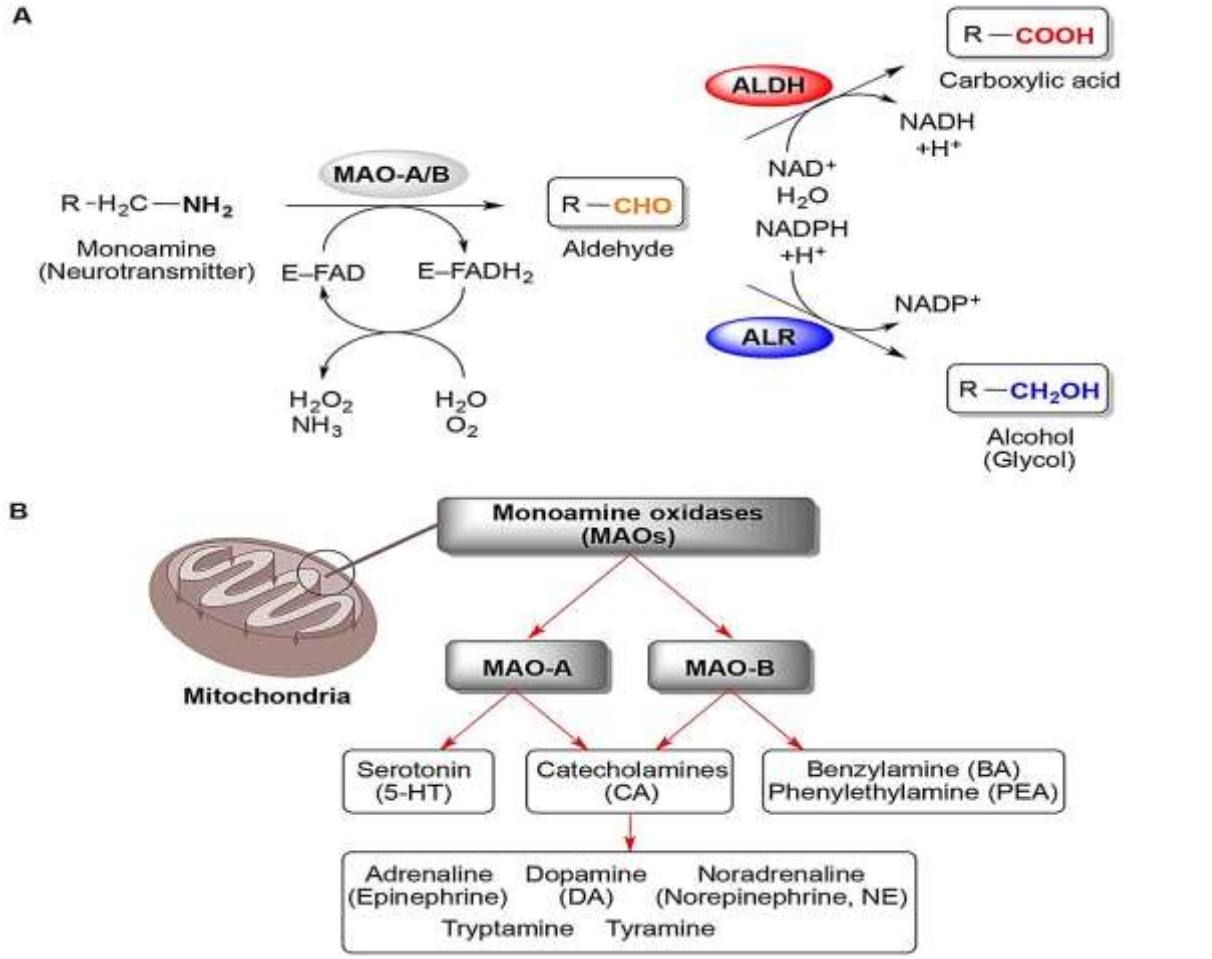



**Fig. 4: Oxidative deamination of monoamines catalyzed by monoamine oxidases (MAOs) A&B**

**INDOLEAMINE 2,3-DIOXYGENASE**

The kynurenine pathway of tryptophan metabolism is initiated by the activity of indoleamine-2,3-dioxygenase (IDO) enzyme. Degradation of tryptophan by these rate-limiting enzymes induces the formation of kynurenine as an intermediate substrate, which can be converted to other catabolic products of the two distinct routes, either potentially neuroprotective or neurotoxic (Figure 5). [19]

The neuroprotective pathway is facilitated by kynurenine aminotransferase, which converts kynurenine into kynurenic acid. Kynurenic acid is an endogenous N-methyl-D-aspartate (NMDA) receptor antagonist, glutamate receptor blocker, and potentially neuroprotective metabolite. The neurotoxic pathway leads to the transformation of kynurenine into 3-hydroxykynurenine catalysed by kynurenine 3-monooxygenase or kynurenine 3-hydroxylase. The inhibition of IDO can decrease kynurenine synthesis and increase serotonin synthesis pathway. In animal models of depression, IDO has been shown as a critical molecular mediator of inflammation induced depressive-like behaviour. [19,20]

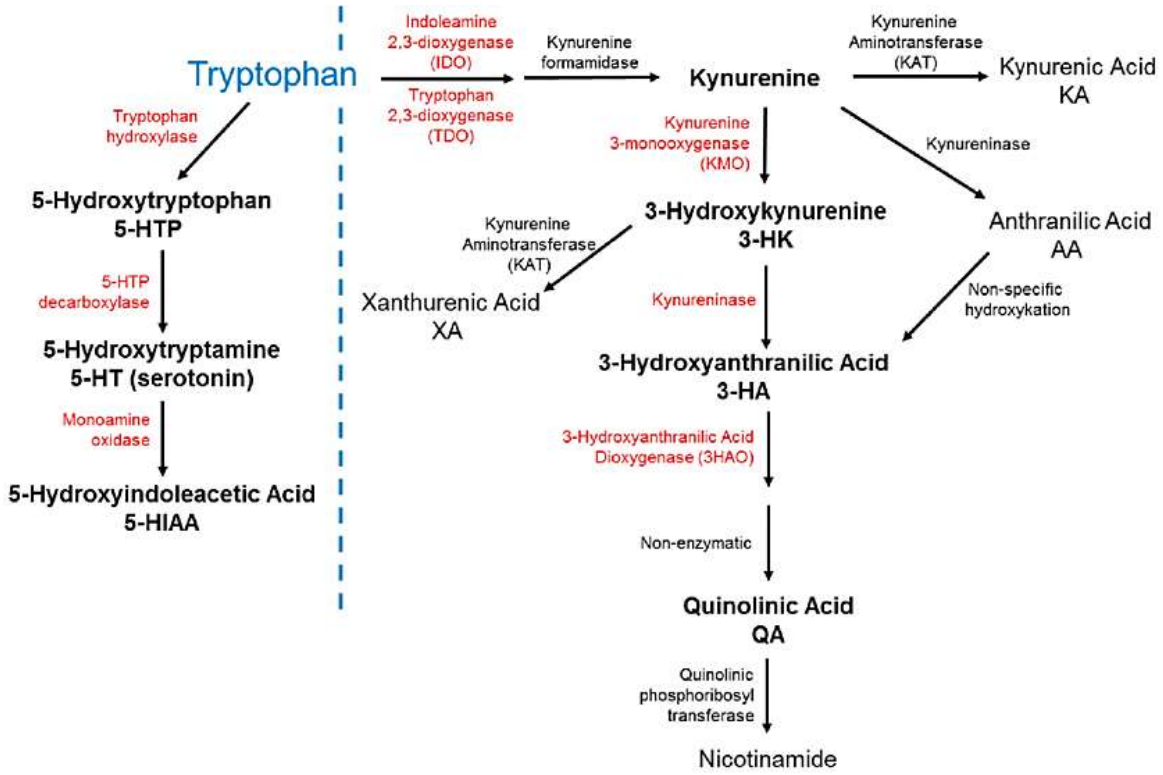



**Fig: 5. Kynurenine pathway of Tryptophan metabolism**

## MANAGEMENT OF DEPRESSION

An array of treatment options has been developed to combat depression over the decades. The various approaches include pharmacotherapy, psychotherapy and somatic therapy often employed for treatment resistant depression. [21]

### Pharmacotherapy

The first anti-depressants were discovered by serendipity, following incisive clinical observations that iproniazid, a drug developed for the treatment of tuberculosis, showed mood elevating effects. Just as well, imipramine, an alleged antipsychotic drug showed antidepressant activity. These observations not only led the way for subsequent studies to develop the first groups of antidepressant drugs MAO inhibitors and TCAs, but also have contributed immensely to the pathophysiological understanding of depression as we know it today. [21]

Majority of the available antidepressant drugs work by modulating the brain monoamine neurotransmission. The primary mechanism of these drugs is increasing the overall synaptic concentration of monoamines (serotonin, norepinephrine and dopamine). They achieve this either by blocking their reuptake into the presynaptic neuron by binding to the respective neurotransmitter transporter or through inhibition of the monoamine degrading enzyme MAO reversibly or irreversibly. Certain antidepressants also act on presynaptic or postsynaptic neurotransmitter receptors to alter the neurotransmission. There also atypical antidepressant drugs that are emerging in the market. This list includes antipsychotics, NK-1 antagonist, GR antagonists and melatonergic drugs. [21,22]

As mentioned earlier, there is a time delay on the onset of the response after treatment with antidepressants. It is believed that long term neuronal adaptations may underlie the effects rather than the acute modulation of transporters or receptors that alter the neurotransmission. Repetitive activation of the neurons by these drugs is believed to result in changes such as synaptic plasticity, axonal sprouting, neurite extension, and promotion of cell survival cue brought about by complex cellular signal transduction mechanisms involving neurotrophins and various transcription factors. [22]

**Somatic therapy**

Somatic therapy for depression is a device-based approach that consists of introducing transient electric or magnetic current onto the scalp or to anatomically deep brain structures. The use of this approach is favored in the management of depression refractory to the available drugs. It also has a wide applicability for maintenance of effect after successful remission as well as can be used as an add-on therapy. [22,23]

The various somatic treatments are believed to induce transient seizures that are responsible for the clinical effects. The mechanism of action is largely attributed to increasing the level of neurotransmitters and sensitization of post synaptic receptors through changing the neuronal firing in the regions involved. There is also the participation of growth factors and induction of long lasting neuronal adaptation. [22,23]

**THERAPIES FOR DEPRESSION** [24]

1. **Reversible inhibitors of MAO-A (RIMAs) -** Moclobemide, Clorgyline, Phenelzine, Iproniazid
2. **Tricyclic antidepressants (TCAs) -** Imipramine, Amitriptyline, Trimipramine, Doxepin, Dothiepin, Clomipramine, Desipramine, Nortriptyline, Amoxapine, Reboxetine, Protiptyline, Maprotiline
3. **Selective serotonin reuptake inhibitors (SSRIs) -** Fluoxetine, Fluvoxamine, Paroxetine, Sertraline, Citalopram, Escitalopram, Dapoxetine
4. **Serotonin and noradrenaline reuptake inhibitors (SNRIs) -** Venlafaxine, Duloxetine
5. **Atypical antidepressants -** Trazodone, Mianserin, Mirtazapine, Bupropion, Tianeptine, Amineptine, Atomoxetine, Nefazodone, Reboxetine.

**MODELS OF DEPRESSION**

Various models of depression have been used to mimic depressive state. Each model has advantages and disadvantages. The most commonly used paradigms are as follows:

**Learned helplessness**

In this paradigm the depressive state is induced by uncontrolled and unpredictable electric shock in rodents. Model of learned helplessness was firstly used at the early 60's. In these

experiments, animals are repeatedly exposed to aversive stimuli without possibility of escape. The animals discontinue attempts to avoid stimuli. As a consequence, when finally, possibility to escape becomes available, they do not perform any action. [25]

**Social defeat stress**

This model shows that the majority of stressful stimuli leading to psychopathological changes have a social nature. In this paradigm, it is possible to study the social stress that consequently leads to depressive-like state in rodents. [25]

**Chronic Unpredictable Mild Stress (CUMS)**

This model intends to use chronic stress, which leads to the depressive state. CUMS is now the most useful model of depression. It is widely adapted rodent paradigm, used to induce depressive and anxiety-like behaviours. It imitates stressful situations of peoples' everyday life. Impulses that initiate the stress response in laboratory animals, so-called stressors, act usually from two to four weeks, and are potentially harmless to the body. CUMS-induced cognitive impairments and anxiogenesis are also reversed by chronic antidepressant treatment. Unpredictable is more likely to use random stressor presentation. [26]

Stressors used in the CUMS model include, among others, food and water reduction, swimming in hot and cold water, wet litter, squeezing the tail, temperature changes, sounds of predators, cage tilt of 45°, changes in light-dark cycle, the presence of rat droppings in the mouse cages etc. [26]

**CHRONIC UNPREDICTABLE MILD STRESS (CUMS)**

Animal models of psychiatric states are procedures applied to laboratory animals which engender behavioural changes that are intended to be homologous to aspects of psychiatric disorders, and can therefore be used as experimental tools to further the understanding of human psychopathology. The chronic unpredictable mild stress (CUMS) model of depression is often considered as a prototypical example. Chronic unpredictable mild stress (CUMS) induced depression model was used, since it imitates the stressful situation in people's day-to-day life. In this model, rats or mice are exposed chronically to a constant bombardment of unpredictable micro-stressors, resulting in the development of a plethora of behavioural changes, including decreased response to rewards, a behavioural correlate of the clinical core symptom of depression and anhedonia. The origin of the CMS model was in series of studies by Katz and colleagues, published in the early 1980s, in which rats were exposed sequentially to a variety of severe stressors. [25,27]

The behavioural changes along the alteration in endocrine and neural variables are similar to those found in individuals suffering from a major depressive disorder and this condition, is reversed by only chronic treatment with antidepressant drugs. The term 'Unpredictable' denotes that the procedure is more random vs fixed stress presentation, and whether the term 'Mild' denotes that the procedure is less severe. The effects of CUMS include, in addition to anhedonia-like impairments in tests of rewarded behaviour, decreases in the performance of other motivated (e.g. sexual and aggressive) behaviours, relative weight loss (i.e., a slower rate of weight gain), disrupted sleep patterns, decreased locomotor activity, and decreased "active waking" in the EEG, all of which parallel symptoms of major depression, as well specific characteristics of melancholia, such as worsening in the early part of the waking phase and a phase advance of circadian rhythms. [27,28]

The CUMS model displays face-predictive and construct validity and hence considered as one of the most useful tests to induce depression and also to study of the effect of chronic treatment with antidepressants. The CUMS procedure uses the application of unpredictable mild stressors that are actually designated to mimic the day to day problems encountered by humans, which in turn have been reported to contribute to the onset of depression. [28]

## *IN SILICO* STUDIES

### DRUG DESIGN

Drug design is a process which involves the identification of a compound that displays a biological profile and ends when the biological profile and chemical synthesis of the new chemical entity are optimized. Drug designing is otherwise known as rational drug design and it is a method of finding new medications based on the biological receptors and target molecules. It involves the designing of small molecules which is complementary to the biological receptor to which they bind and interact to cause the pharmacological actions. Modern method of drug designing is done with the aid of computers and hence, the process is known as Computer Assisted/Aided Drug Design (CADD). It uses computational chemistry to study about the drugs and related biologically active molecules. The major aim is to find whether the given molecule bind to the target and causes pharmacological actions or not. The basic steps involved in CADD are:

1. Hit identification using virtual screening.
2. Hit-to-lead optimization of affinity and selectivity.
3. Lead optimization of other pharmaceutical properties maintaining affinity. [29]

**Types of drug design**

- Ligand based drug design
- Structure based drug design

**Ligand based drug design**

It is also known as indirect drug design. In the absence of the structural information of the target, ligand-based method is used to know about inhibitors for the target receptor. Biologically active lead molecule is detected by using structural or topological similarity or pharmacophoric similarity properties. There are several criteria for similarity comparisons such as structure as well as shape of individual fragment or electrostatic properties of the molecule. The generated lead molecules are ranked based on their similarity score or obtained by using different methods or algorithms. [30]

**Structure based drug design**

It is also known as direct drug design. It depends on the knowledge of 3D structure of the biological target that is obtained via methods such as X-ray crystallography, NMR spectroscopy or homology modelling. It is used to create a homology model of target when the experimental structure is unavailable. With the three-dimensional structure of the target obtained from X-ray crystallography or NMR spectroscopy, one can begin the search for a ligand whose orientation and conformation are complimentary to the receptor structure. Therefore, structures of the target molecules have to be decided and the exact ligand molecule need to be designed. [31]

Steps involved in drug design - The drug design process may be categorized into following four distinct stages.

1) Selection and identification of the target.
2) Search for lead or lead identification.
3) Lead optimization.
4) Synthesis of new molecules.

**Selection and identification of the target**

Drug discovery process begins with the identification of a possible therapeutic target. The selected drug target must be a key molecule involved in a specific metabolic or cell signalling pathway that is known or believed to be related to a particular disease state.

Important drug targets include: Enzymes (inhibitor- reversible or irreversible), receptors (agonist or antagonist), nucleic acid intercalators or modifiers, Ion channels (blockers or openers), transporters (uptake inhibitors). [31]

The 3D structure of the protein target is usually obtained by X-ray crystallography [crystal structures of different macromolecules are available from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Database], NMR or homology modelling from a previously determined structure. [32]

**Search for lead (lead identification)**

Lead structures are ligands which are selected from a series of related compounds that exhibit suboptimal target binding affinity. After lead selection, they are tested for their activity towards a desired drug target. [32,33]

a) *Denovo* molecular design

b) Database search methods

c) Combinatorial methods

**Lead optimization**

Lead optimization is a process in which lead compounds are altered to make them more effective and safer i.e., to achieve maximum affinity to the target with improved bioavailability and low toxicity. By effective combination of two or more active moieties or by elimination or substitution of various groups, the properties of the lead compound can be modified. [33]

**Drug targets**

Target is a naturally existing cellular or molecular structure involved in the process of drug development and meant to act on. Already established targets help in understanding of both how it is involved in human pathology and how the target functions in normal physiology. The new targets are not established targets. New targets include newly discovered protein molecules, and those proteins functions have been clear under scientific research process. [31,32]

**Identification of right target and active site**

Target is a protein molecule and it closely related to disease. It plays an important role in signal transduction pathway that often disrupted in the diseased condition. Enzymes are one of the targets, they have binding pockets for inhibition as well as substrates. [31]

**Drug likeness**

Drug likeness may be defined as a complex balance of various molecular properties and structural features which in turn determine whether a particular molecule is similar to the known drugs. In silico evaluation of drug likeness at an early stage involves the prediction of various ADMET (absorption, distribution, metabolism, excretion, toxicity) properties using computational approaches, i.e., it provides a preliminary prediction of the in vivo behaviour of a molecule. Drug-likeness score towards GPCR ligands, ion channel modulators, kinase inhibitors, nuclear receptor ligands and protease inhibitors may be evaluated online by using 'Molinspiration server'. The higher the value of the score, the more the probability that the particular molecule will be active. [31]

**Lipinski's rule of Five**

This rule was postulated by Christopher A. Lipinski in 1997, based on the observation that most of the drugs are relatively small and lipophilic in nature. Lipinski's rule of five is a rule of thumb to evaluate drug likeness. This rule states that, an orally active "drug-like" molecule has:

- Partition coefficient (log P) less than 5
- Molecular weight under 500 Daltons
- Not more than 10 hydrogen bond acceptors (O and N group)
- Not more than 5 hydrogen bond donors (OH and NH group)
- Number of violations less than 2

All the numbers should be multiples of 5, which is the basis for the rules name. The rule describes about pharmacokinetic properties of a drug in the human body, including their absorption, distribution, metabolism, and excretion (ADME). But this rule does not predict if a compound is pharmacologically active or not. This set of rules suggests that the necessary properties for good oral bioavailability and reflects the notion that pharmacokinetics, toxicity and other adverse effects are directly linked to the chemical structure of a drug. [34]

**ADMET properties**

The purpose of preclinical ADME, also referred to as early drug metabolism and pharmacokinetics (DMPK), is to reduce the risks and avoid spending valuable resources on molecules that have poor pharmacokinetic (PK) properties. This strategy allows drug discovery resources to be focused on fewer but higher-quality drug candidates. Since then, major technological advances have occurred in molecular biology and screening, which allow major aspects of ADME to be assessed earlier during the lead optimization stage. Bioavailability, tissue distribution, pharmacokinetics, metabolism, and toxicity are assessed typically in one rodent and one non-rodent species prior to administering a drug to a human to evaluate drug pharmacokinetics and exposure in a clinical trial. The progress of ADME profiling has decreased the proportion of drug candidates failing in clinical trials for ADME reasons and providing important early input into safety and toxicity prediction of drug candidates. [35]

**MOLECULAR DOCKING**

Docking is a method which predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex. Preferred orientation helps to predict the strength of association of or binding affinity between two molecules. The associations with biological molecules such as proteins, nucleic acids, carbohydrates and lipids play an important role in signal transduction i.e. agonism or antagonism. So docking is a useful tool for predicting both the strength and type of signal produced. [35]

Molecular docking may be defined as an optimization program, which would describe the 'best-fit' orientation of a ligand that binds to a particular protein of interest. The focus of molecular docking is to computationally stimulate the molecular recognition process. The aim of molecular docking is to achieve an optimized conformation for both the protein and ligand and relative orientation between protein and ligand such that the free energy of the overall system is minimized. [36]

A molecular docking calculation consists of the following steps:

1. Optimization of the ligand geometry, calculation of pH-dependent partial charges, and identification of rotatable bonds.

2. Calculation of electrostatic properties of the protein of interest and defining the

ligand –binding region.

3. Calculation of ligand-protein interaction by a scoring function that includes terms and equations that describe the intermolecular energies.

Docking produces plausible candidate structures. These candidates must be ranked by using scoring functions and to identify structures that are most likely to occur in nature. [37]

**Rigid-body docking and flexible docking**

If the bond angles, torsion angles and bond lengths of the components are not modified at any stage of complex generation, then they are known as rigid body docking. A rigid-body docking is sufficiently good for most docking, when substantial change occurs within the components at rigid-body docking. Docking procedures which permit flexible docking procedures or conformational change, must intelligently select small subset of possible conformational changes for consideration. [37]

Docking approaches - Two approaches mainly popular in molecular docking.

Shape complementarity - This technique is used to describe the matching of ligand and protein as complementarity surfaces.

Simulation - It is the actual docking process additionally calculating the interaction and energies between ligand and protein molecule. [36,37]

**Softwares for Docking**

Over 60 docking software systems and more than 30 scoring functions are reported. Molecular docking is implemented as part of software packages for molecule design and simulation. More than one search method and scoring functions are provided in order to increase the accuracy of the simulations. Only some of the software was made available and a limited number of them are widely used [38]. Commonly used software's are: AutoDock, Gold, Vega, Glide, FlexiDock, Flex, Fred, Hint etc. [39]

**AutoDock 4.2**

Autodock is a suite of automated docking tools. It is designed to predict how small molecules, such as substrates or drug candidates, bind to a receptor of known 3D structure. Autodock uses Monte Carlo method and simulated annealing in combination with genetic algorithm for building the possible conformations.

The genetic algorithm is used for global optimization. Autodock works in Linux platform. Cygwin is used as a user-friendly interface. The local search method is energy minimization and Amber "force field" model helps in the evaluation of binding positions compatible with several scoring functions based on the free energy. The atomic affinity grids can be visualized. This is helpful to guide organic synthetic chemists to design better binders. Autodock consists of two main programs:

- AutoGrid pre-calculates the grids
- AutoDock perform the docking of the ligand to a set of grids describing the target protein.

It also has got capabilities to visualize atomic affinity grids and its graphical user interface, thus to support the analysis of docking results. It has an advantage of getting free academic license, at the same time parallel computation is not supported. [39]

**DRUG PROFILE**

**Compound name** – Isatin

**IUPAC name** - 1*H*-indole-2,3-dione

**Formula** - $C_8H_5NO_2$

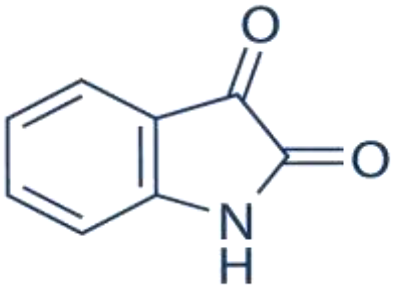



**Fig. 6: Structure of Isatin**

**Molar mass** – 147.1308 g/mol

**Melting point** - 200°C (473 K)

**Solubility**- Soluble in hot water, hot ethanol, benzene, acetone, ether. Partially soluble in organic solvents such as Dichloromethane or Chloroform. Insoluble in nonpolar organic solvents such as hexanes, benzene, and toluene. [40]

**Appearance** - Orange-red solid

**Reported activities** – Anti-depressant [41,43,44], anti-microbial [41], anti-biotic [41,45], anti-inflammatory [41], analgesic [42], anti-convulsant [42,44], anti-malarial [42], anti-cancer [42], anti-ulcer [42], anti-leishmanial [42,45], contraceptive and anti-oxidant activities [42,45].

## FUNDAMENTAL REACTIVITY OF ISATIN

Isatin or 1H-indole-2, 3-dione (Fig. 6) is an indole derivative. The compound was first obtained by Erdman and Laurent in 1841 as a product from the oxidation of indigo dye by nitric acid and chromic acid. The presence of several reaction centers in isatin and its derivatives render them capable of participating in a large number of reactions. The keto group at position 2 and particularly at position 3 can enter into addition reactions at the C-O bond and into condensation reactions. [45,46]

In nature, isatin is found in plants of the genus *Isatis*, in *Calanthe discolor* Lindl. and in *Couroupita guianensis* Aubl., and has also been found as a component of the secretion from the parotid gland of *Bufo* frogs, and in humans as it is a metabolic derivative of adrenaline. Substituted isatins are also found in plants, for example the melosatin alkaloids (methoxy phenylpentyl isatins) obtained from the Caribbean tumorigenic plant *Melochia tomentosa* as well as from fungi: 6-(3'-methylbuten- 2'-yl) isatin was isolated from *Streptomyces albus* and 5- (3'-methylbuten-2'-yl) isatin from *Chaetomium globosum*. [47]

### Biological Activities of Isatin and its derivatives

The indole ring is found in many naturally occurring compounds, notably alkaloids, fungal metabolites and marine natural products. Indole derivatives have been found to possess several biological properties, including anti-microbial, anti-biotic, anti-inflammatory, analgesic, anti-convulsant, anti-malarial, anti-cancer, anti-ulcer, anti-leishmanial, contraceptive and antioxidant activities. Isatin itself is known to possess CNS-MAO inhibition, anti-convulsant and anxiogenic activities. Isatin derivatives show such diverse activity, including fibrinolytic, muscle relaxant, anti-allergic, immunosuppressant, and antithrombotic activity. Isatin derivatives also have agonistic effects on several receptors. In particular, 3-substituted 3-hydroxyoxindole has been found to be a structural motif in alkaloid based natural and synthetic pharmaceuticals. [43-46]

**STRUCHURE ACTIVITY RELATIONSHIP**

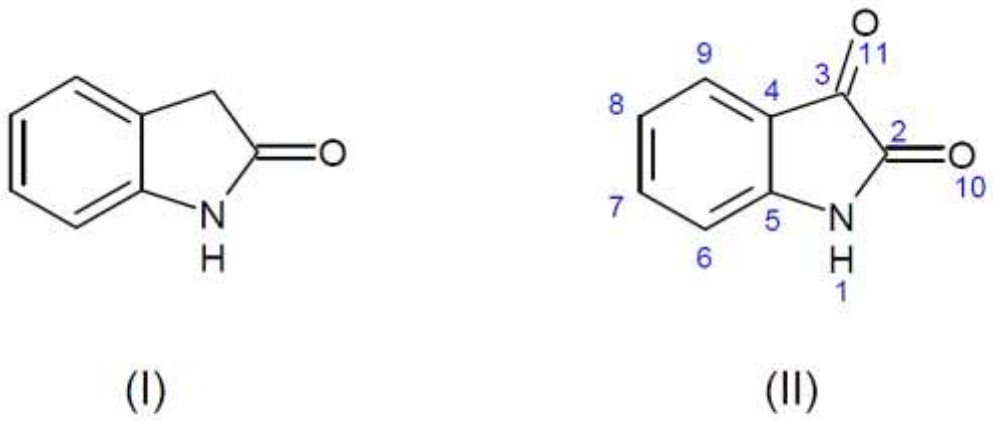



**Fig 7: Structure activity relationship of isatin**

1. Bond acceptor at the position 3
2. Free rotation bond O-H
3. Bond donor at position 1
4. Polar surface area -37.38
5. C5, C6 and C7 substitution generally enhanced CNS activity with some di and tri halogenated isatin (Fig.7). [48]

**MAO inhibitor activity**

Medvedev *et al*., found that indole and 2, 3-dioxindole analogues an inhibitor of MAO-A and B. The compounds exhibited reversible and competitive MAO inhibition. The substituents at C-2 and C-3 of indole ring for selective MAO-A inhibitor. The presence of hydroxyl group at C-5 of isatin increased selectivity of MAO-A inhibitor. However, simultaneous insertion of substituents into both positions of indole ring (5-hydroxy-2-phenylindole) led to a decrease of MAO-A inhibitor. he MAO-B inhibition also depended on the sizes of planar molecules. However, distribution of electron density in the molecules was another precondition for the selective inhibition of the enzyme. Halogens at position 5, 6, and 7 increase the activity of isatin derivatives compound. [49]

From literature that isatin containing synthetic compounds and their derivatives are known to be associated with broad spectrum of biological activity like antipyretic activity, analgesic effect, anticonvulsant activity, few compounds were also reported as psychotropic agents and MAO inhibitors. [48]

**REVIEW OF LITERATURE**

**Tong *et al.*, (2019)** investigated the effect of helicid on depression related behaviours in rats exposed to chronic unpredictable mild stress (CUMS) and have also explored possible underlying mechanisms that involve neurotrophin expression. After 6weeks isolation, body weight and sucrose preference will be significantly reduced in rats with CUMS-induced depression compared with controls. Helicid also reversed CUMS-induced decreases of 5-HT1A receptor expression and promoted brain derived neurotrophic factor (BDNF) expression in the hippocampus. [50]

**Song *et al.*, (2018)** has reported anti-inflammatory and anti-oxidant activity in Perilla aldehyde (PAH). The aim of this study is to explore the antidepressant-like effect and the underlying mechanism of PAH, a major constituent from *Perilla frutescens*, on the rat model induced by CUMS. The treatment with PAH and fluoxetine significantly improved the sucrose consumption, immobility time in forced swim test, as well as locomotor activity in open-field test. The levels of pro-inflammatory cytokines in hippocampus will be also suppressed. PAH exhibited antidepressant-like effect in CUMS-induced rat model of depression, which might be mediated by TXNIP/TRX/NLRP3 pathway. [51]

**Cavusoglu *et al*., (2018)** synthesized the new series of thirteen 2-[(4-fluorophenyl) (4-nitrobenzyl)amino]-2-oxoethyl-1-substituted-carbodithioate derivatives (4a-4m) and tested for their human monoamine oxidase A and B (hMAO-A and hMAO-B) inhibitory potential by an *in vitro* fluorometric method. Most of the compounds have found to be selective towards MAO-B than MAO-A. Compound 4j that carrying 4-nitrophenyl piperazine moiety, was detected as the most active agent amongst all compounds with the $IC_{50}$ value of $0.097 \pm 0.003$ μM for MAO-B while that of selegiline was $0.040 \pm 0.002$ μM. The enzyme kinetic study reported that compound 4j is a reversible and non-competitive inhibitor. Interaction modes between the hMAO-B and compound 4j were determined by docking studies. The study also revealed that compound 4j has the highest binding scores. [52]

**Kerzare *et al.*, (2018)** has made an attempt to generate new molecular template by linking two pharmacophores (indole and azetidinone), which are likely to produce antidepressant-like action in animal models. The evaluation of pharmacological potential of the indole bearing azetidinone derivatives as antidepressant agent has been relatively less explored. To get insight of the intermolecular interactions, the molecular docking studies are performed at active site of MAO-A enzyme. The derivatives were synthesized by conventional

reactions and characterized by various spectrometric methods. Molecular docking studies of the synthesized derivatives with MAO-A enzyme was performed. Compounds showed enough potential to be developed as antidepressant agent. [53]

**Ogyu *et al*., (2018)** reviewed the abnormalities of the kynurenine (KYN) pathway and its implication in the pathophysiology of depression. However, the relationships between depression and each metabolite of the KYN pathway remain uncertain. Kynurenic acid (KYNA) and KYN levels were lower in patients with depression in comparison to controls, while quinolinic acid (QUIN) levels did not differ between the two groups. Antidepressant-free patients showed decreased KYNA levels and increased QUIN levels compared with controls. Male ratios of the samples were negatively associated with study of KYNA. In conclusion, this analysis revealed that patients with depression had decreased level of KYNA and KYN, whereas antidepressant-free patients showed increased level of QUIN. [54]

**Griglio *et al*., (2018)** reported that indoleamine 2,3-dioxygenase plays a crucial role in immune tolerance and has emerged as an attractive target for cancer immunotherapy. In this study, multicomponent reactions have been employed to assemble a small library of imidazothiazoles that target IDO1. While the p-bromo phenyl and the imidazothiazole moieties have been kept fixed, a full SAR study has been performed on the side-chain, leading to the discovery of nine compounds with sub-micromolar $IC_{50}$ values in the enzyme- based assay. Molecular docking studies show that both 7d and 6o display a unique binding mode in the IDO1 active site, with the side-chain protruding in an additional pocket C, where a crucial hydrogen bond is formed with Lys238. Overall, this work describes an isocyanide based-multicomponent approach as a straightforward and versatile tool to rapidly access IDO1 inhibitors, providing a new direction for their future design and development. [55]

**Suresh *et al*., (2017)** reported Indoleamine 2, 3-dioxygenase (IDO) as an emerging new therapeutic drug target characterized by pathological immune suppression. The study focuses on the design of 1H-Indole-2, 3-Dione analogues containing triazole, followed by docking against the known anticancer targets like indoleamine 2, 3-dioxygenase (IDO) and EGFR tyrosine kinase. Twenty-five compounds of 1H-Indole-2, 3-Dione analogues were designed and docked against indoleamine 2, 3-dioxygenase (IDO) and EGFR tyrosine kinase using Auto dock (version 4.2). Among the docked compounds five compounds (Tri1, Tri6, Tri12, Tri16, and Tri20) showed a significant docking score against target enzyme compared to the standard.

The study concludes that 1H-Indole-2, 3-Dione analogues will be a significant lead for further investigation of anti-cancer properties. [56]

**Ma *et al.*, (2016)** designed, synthesized, and screened a series of 3-phenyliminoindolin-2-one derivatives for their antidepressant and anticonvulsant activities. The IR spectra of the compounds afforded NH stretching (3340–3346 $cm^{-1}$) bands and C=O stretching (1731– 1746 $cm^{-1}$). In the $^{1}$H-NMR spectra of the compounds, N-H protons of indoline ring were observed at 10.65– 10.89 ppm generally as broad bands, and $^{13}$C-NMR spectra of the compounds C=O were seen at 161.72– 169.27 ppm. [57]

**Joubert *et al.*, (2016)** designed novel multifunctional neuroprotective agents that slow down or halt neurodegeneration by inhibiting MAO-A, MAO–B and caspase-3. The synthesised compounds consisted of isatin nucleus conjugated to a fluorophenylsulfonyl moiety at position 5 and on selected compounds a propargylamine moiety was introduced at the N-position of isatin. All the compounds showed promising MAO-A inhibitory activity. The compounds without the propargylamine moiety showed better MAO-B inhibition. The active compounds showed improved inhibitory activities when compared to the reference compounds. Possible binding orientations of selected isatin analogues within the active site cavities of MAO-A, -B and caspase-3 were proposed and in silico ADMET studies were also performed. [58]

**Badavath *et al.*, (2016)** designed and synthesized series of new 2-methoxy-4-(5-phenyl-4,5- dihydro-1H-pyrazol-3-yl) phenol derivatives, 4−13, and tested for their human MAO inhibitory activity. All the compounds were found to be selective and reversible toward hMAO-A except 4, a selective inhibitor of hMAO-B and 12, a nonselective inhibitor. Compound 7 was found to be a potent inhibitor of hMAO-A with Ki = 0.06 ± 0.003 μM and was having selectivity index of (SI = 1.02 × 10−5). It was found to be better than standard drug, Moclobemide (hMAO-A with Ki = 0.11 ± 0.01 μM) with selectivity index of SI = 0.049. Molecular docking simulation was carried out to understand the crucial interactions responsible for selectivity and potency. [59]

**Dias *et al.*, (2016)** detailed the activation of the enzyme indoleamine-2,3-dioxygenase (IDO), that participate in the tryptophan metabolism leading to decrease of serotonin (5-HT) levels and whose expression is associated with an immune system activation, has been proposed as a common mechanism that links depression and diabetes. To test the hypothesis, diabetic (DBT) and normoglycemic (NGL) groups had the cytokines (TNFα, IL-1β, and IL-6)

and 5-HT and norepinephrine (NE) levels in the hippocampus (HIP) were evaluated. The effect of the selective serotonin reuptake inhibitor fluoxetine (FLX), IDO direct inhibitor 1-methyl-tryptophan (1-MT), anti-inflammatory and IDO indirect inhibitor minocycline (MINO), or non-selective cyclooxygenase inhibitor ibuprofen (IBU) was evaluated in DBT rats exposed to the modified forced swimming test (MFST). DBT rats exhibited a significant increase in HIP levels of TNFα, IL-1β, and IL-6 and a decrease in HIP 5-HT and NA levels. They also presented a depressive-like behavior which was reversed by all employed treatments. [60]

**Suthar *et al*., (2015)** designed, synthesized, and evaluated antidepressant activity of 3-substituted oxindole derivatives by employing forced swimming test, tail suspension test, and MAO-A inhibition assay. Results of biological studies revealed that the majority of compounds exhibited potent to moderately potent activity and among them, 12 displayed potency comparable to that of the imipramine with %DID of 37.95 and 44.84 in the FST and TST, respectively. In the MAO-A inhibition assay, 12 showed an $IC_{50}$ of 18.27 µmol, whereas the reference drug moclobemide displayed an $IC_{50}$ of 13.1 µmol. The SAR study revealed that the presence of bromo atom at the phenyl/furanyl or thienyl moiety in the oxindole derivatives was crucial for the antidepressant activity. [61]

**Debnath *et al*., (2015)** reported the synthesis and evaluation of some isatin analogs for anthelmintic activity against *Pheritima posthuma*. A series of 16 isatin analogs (3a-3p) were synthesized, characterized by physical and spectral data (Fourier transform infrared,1H nuclear magnetic resonance and mass) and screened for anthelmintic activity against *P. posthuma* at various concentrations (5, 10, and 20 mg/ml). All compounds were tested for the beginning of paralysis time followed by time of death of the worms. All prototypes at the concentration of 20 mg/ml showed significant activity ($p<0.01$) compared to that of control. The compounds 3f and 3i were found to possess significant activity compared to standard albendazole (20 mg/ml).[62]

**Liu *et al*., (2015)** investigated the effects of icariin on the depression-like behaviors in an unpredictable chronic mild stress (CMS) model of depression in rats. Rats exposed to CMS showed behavioral deficits in physical state, the sucrose preference test (SPT) and the forced swimming test (FST) and exhibited a significant increase in oxidative–nitrosative stress markers, inflammatory mediators, including tumor necrosis factor-alpha (TNF-a) and interleukin-1b (IL-1b), activation of the nuclear factor kappa B (NF-jB) signalling pathway and increased inducible nitric oxide synthase (iNOS) mRNA expression in the hippocampus, which

was reversed by chronic treatment with icariin (20 or 40 mg/kg). The study demonstrates that CMS produces depression-like behavior and causes changes in the oxidative stress and inflammatory response in the hippocampus of rats and that icariin attenuates or reverses these effects, which may be mediated, at least in part, by increased antioxidant status and an anti-inflammatory effect in the brain tissue via inhibition of NF-jB signalling activation and the NLRP3- 694 inflammasome/caspase-1/IL-1b axis. [63]

**Farhan A *et al.*, (2015)** reported the pharmacological activities of isatin like anti-allergic, antimalarial, antiviral and antimicrobial; isatin and its derivatives have been found to show promising results against various cancer cell lines. Isatin is a versatile precursor for many biologically active molecules and its diversified nature makes it a versatile substrate for further modifications. Isatin is a synthetically versatile molecule with a diversified nature of pharmacological applications. [64]

**Mishra (2014)** has carried out the synthesis and biological studies of some isatin (indole 2,3-diones). Fifty-seven derivatives were prepared and substitutions were made at positions 1 and 3. The isatin derivatives (MP $V_{A-F}$) were prepared by the substitution of different aldehydes by reacting the mixture of (3Z)-3-[(4-hydrazinylphenyl) imino]-1,3-dihydro-2H-indol-2-one in the presence of glacial acetic acid. Isatin derivatives with acid anhydride were prepared and isatin derivatives were also reacted with a yield of 85%. All the new compounds were then screened for their antimicrobial activity against test organism. [65]

**Grewal *et al.*, (2014)** performed docking simulations on the series (Hetero) arlylidene-(4-substituted-thiazol-2-yl) hydrazines as MAO-B inhibitors. This was done by analysing the interaction of these compounds with the catalytic site of the MAO-B enzyme. Assuming that the enzyme inhibition is a function of the interaction energy, from a comparison with pIC50 a good correlation between theoretical and experimental data was observed. This suggests that identified binding conformations of these inhibitors are reliable. The results of docking studies provide an insight into the pharmacophoric structural requirements for the MAO-B inhibitory activity of these class molecules. [66]

**Pakravan *et al.*, (2013)** provided an overview of the pharmacological activities of isatin and its synthetic and natural derivatives. Molecular modifications to tailor the properties of isatin and its derivatives are also discussed. Isatin is a heterocyclic compound of significant importance in medicinal chemistry. It is a synthetically versatile molecule, a precursor for a large number of pharmacologically active compounds. Isatin and its derivatives have aroused

great attention in recent years due to their wide variety of biological activities, relevant to application as insecticides and fungicides and in a broad range of drug therapies, including anticancer drugs, antibiotics and antidepressants. [67]

**Kim *et al*., (2012)** reported that brain indoleamine 2,3-dioxygenase 1 (IDO1), a rate-limiting enzyme in tryptophan metabolism, plays a key role in pain and depression. It was found that chronic pain in rats induced depressive behavior and IDO1 upregulation in the bilateral hippocampus. Upregulation of IDO1 resulted in increased kynurenine/tryptophan ratio and decreased serotonin/tryptophan ratio in the bilateral hippocampus. Intra-hippocampal administration of IL-6 in rats, in addition to *in vitro* experiments, demonstrated that IL-6 induces IDO1 expression through the JAK/STAT pathway. In addition, either IDO1 gene knockout or pharmacological inhibition of hippocampal IDO1 activity attenuated both nociceptive and depressive behavior. These results reveal an IDO1-mediated regulatory mechanism underlying the comorbidity of pain and depression. [68]

**Castagne *et al.*, (2011)** reported about the rodent models of depression widely used in screening tests used for antidepressants, the forced swim (also termed behavioral despair) test in the rat and mouse, and the tail suspension test in the mouse. These tests have good predictive validity and allow rapid and economical detection of substances with potential antidepressant-like activity. The behavioural despair and the tail suspension tests are based on the same principle: measurement of the duration of immobility when rodents are exposed to an inescapable situation. The majority of clinically used antidepressants decrease the duration of immobility. Antidepressants also increase the latency to immobility, and this additional measure can increase the sensitivity of the behavioral despair test in the mouse for certain classes of antidepressant. Testing of new substances in the behavioral despair and tail suspension tests allows a simple assessment of their potential antidepressant activity by the measurement of their effect on immobility. [69]

**AIM AND OBJECTIVES**

Depression has a high lifetime prevalence of 21% and it is one among the severe psychiatric disorder. Lower rates of fertility can occur with depression. Major depressive disorder is a chief cause of disability worldwide. Women are about twice as likely to suffer from a major depressive event as men. In a year, about 1 million lives are lost due to suicide i.e. 3000 suicide deaths every day. This lethal disorder can be treated with antidepressants. [70]

Stress is one of the causative agents of depression which suppresses BDNF synthesis in the hippocampus of the brain. In depressed patient serum BDNF concentrations are low correlating with the severity of the depression. Major Depressive Disorder is associated with elevated cortisol levels, and chronically elevated levels of CRH. CRH is widely distributed in the brain and has behavioural effects that are distinct from its endocrine functions. CRH mimics some effects of depression in humans, such as diminished activity, loss of appetite, and increased signs of anxiety. Therefore, CRH hyperfunction, as well as monoamine (Serotonin, nor-adrenaline and dopamine) hypofunction, may be associated with depression. The enzyme MAO is responsible for degradation of monoamines. By inhibiting this enzyme, the level of monoamines in neurons will get increased. The enzyme IDO is responsible for conversion of tryptophan to kynurenine. Inhibition of IDO leads to shift to serotonin pathway from kynurenine pathway. Henceforth, the level of serotonin gets increased. [71]

Isatin is a versatile chemical building block, able to form a large number of heterocyclic molecules. The compound possesses an indole ring structure, common to many pharmaceuticals. Isatin itself possesses an extensive range of biological activities. Pharmacological studies have confirmed the use of isatin in different central nervous system (CNS) diseases. It is able to participate in a broad range of synthetic reactions, leading to its extensive use as a precursor molecule in medicinal chemistry. [72]

The present study was designed to evaluate the *in vitro* and *in vivo* antidepressant activity of synthetic derivatives of isatin and their pre-treatment on rats to protect from depression induced by chronic unpredictable mild stress based on *in silico* ADMET and docking scores against MAO and IDO. The present study also attempts to demonstrate the possible mechanism of their therapeutic efficacy by studying the behavioural assessment, neurotransmitter estimation, biochemical estimation and histopathological changes.

The objective of the present work is to evaluate the treatment of depression in rats exposed to chronic unpredictable mild stress using synthetic derivatives of isatin.

**PLAN OF WORK**

**Step 1:** Literature review

**Step 2:** Designing of synthetic derivatives of isatin (SDI).

**Step 3:** Determination of molecular properties and bioactivity scores by using Molinspiration online tool for the derived SDI compounds.

**Step 4:** Evaluate ADMET properties of the SDI by using preADMET online tool.

**Step 5:** To carry out the molecular docking studies of the SDI against MAO and IDO using AutoDock 4.2 software based on the blood brain barrier penetration.

**Step 6:** Selection of two compounds of SDI based on their docking scores.

**Step 7:** Synthesis and purification of two selected compounds of SDI.

**Step 8:** Spectral analysis of two selected compounds of SDI.

**Step 9:** *In-vitro* Monoamine oxidase inhibitor activity.

**Step 10:** Acute toxicity study of two selected compounds of SDI.

**Step 11:** *In vivo* screening of the two selected compounds of SDI by chronic unpredictable mild stress induced depression like behaviour in rats.

**Step 12:** Evaluation of behavioural parameters.

**Step 13:** Euthanasia and collection of blood by retro-orbital plexus puncture method.

**Step 14:** Separation of serum for biochemical estimation.

**Step 15:** Dissection and preparation of brain tissue homogenate.

**Step 16:** Estimation of tissue parameters, histopathological study of brain.

**Step 17:** Tabulation, compilation of results and statistical analysis of data obtained.

**Step 18:** Submission of thesis.

## MATERIALS AND METHODS

### Software and data bases

ChemSketch, Online SMILES translator, PreADMET, Molinspiration, Accerlys discovery studio viewer 4.0.1, RCSB protein data bank, Protein Data Bank in Europe, MGL tools- AutoDock 4.2, Python 2.7 molecule viewer 1.5.6, Vision 1.5.6, Cygwin 64, Visual Molecular Dynamics 1.9.3, GraphPad prism 8, GraphPad instat, Open Babel 2.4.1.

### Reagents and chemicals

Sucrose was purchased from HiMedia chemicals, Coimbatore. Selegiline, Noradrenaline was purchased from Ramakrishna pharmacy, Coimbatore. Serotonin and dopamine was procured from SRL diagnostics, Coimbatore. Dichloromethane, EDTA, iodine, O-phthaldialdehyde, sodium phosphate buffer, bovine serum albumin, trichloro acetic acid, thio-barbituric acid, hydrogen peroxide, Tris-HCL buffer, sodium azide, glutathione, carbonate buffer, Ellman's reagent, potassium phosphate buffer, NADPH, oxidised glutathione was used of analytical grade procured from local suppliers. Isatin was purchased from HiMedia Laboratories, Pennsylvania.

### Instruments

MISPA VIVA Semi auto-analyser, Shimadzu-Jasco V-630 UV – spectrophotometer, MK VI, Jasco FT/IR – 4100, Centrifuge (Remi Instruments Ltd., Kolkata), MR-VIS visual melting range apparatus (LABINDIA), ELCO 1/27 pH meter, Jasco – Spectrofluorimeter.

## *IN SILICO* STUDIES

### Lead and target selection

The present study was focused on antidepressant activity. From the literature review and the current research on depression, we have selected monoamine oxidase and indoleamine 2,3-dioxygenase as the target for the present study. The PDB structure of monoamine oxidase (PDB ID: 2BXR) and indoleamine 2,3-dioxygenase (PDB ID: 6E35) was downloaded from the RCSB protein data bank.

### Evaluation of Drug Likeness Properties – Molinspiration online tool

A total of 35 compounds from derivatives of isatin was drawn using ChemSketch. The SMILES of these compounds was used for determining drug likeliness properties. The theory of drug-likeness score helps to optimize pharmaceutical and pharmacokinetic properties, for example, chemical stability, solubility, distribution profile and bioavailability. The molecular descriptors have developed as rationally predictive and informative, for example, the Lipinski's Rule-of-Five (Ro5). The better oral absorption of the ligands and drug likeness scores will be

constructed by getting information about the solubility, diffusion, Log P, molecular weight etc. Molinspiration software was used to evaluate the Lipinski's rule of five. [73]

**Lipinski's Rule of 5 calculations**

1) Open the Molinspiration home page (http://www.molinspiration.com/).
2) For calculating using Molinspiration, it requires JAVA in the computer.
3) Click calculation of molecular properties of drug likeness.
4) Draw the structure in the active window.
5) Click calculate properties and predict bioactivity.
6) Save the properties.

The compounds showing violations ≤ 2 will be selected and further used for evaluating ADMET properties.

**Evaluation of ADMET properties – *pre*ADMET online tool**

The ADMET studies will be performed for the selected derivatives of isatin using *Pre*ADMET online tool. From this blood brain barrier (BBB), absorption, distribution, metabolism, of the selected ligands will be evaluated. The 2D structure will be directly introduced into *Pre*ADMET to carry out ADMET screening by using ChemSketch software. The data for descriptors is BBB, plasma protein binding, aqueous solubility, skin permeability, hepatotoxicity etc. [38] The compounds possessing high BBB penetration (more than 2.0) will be selected and further evaluated for *in vitro* studies. [74]

***In silico* docking study on monoamine oxidase and indoleamine 2,3-dioxygenase enzyme using AutoDock 4.2**

The compounds which showed optimum BBB penetration from ADMET studies docked against MAO and IDO enzymes.

The compounds which shows better blood brain barrier penetration were selected and docked against then enzymes monoamine oxidase and indoleamine 2,3-dioxygenase using AutoDock 4.2 software. The steps involved in the docking are

- Conversion of refined enzyme into pdb format
- Conversion of pdb format of ligand into pdbqt format
- Preparation of grid box by setting grid parameters
- Docking process by setting docking parameters
- Saving the docked result as dlg file
- Viewing the docked conformation
- Taking snapshots of the interactions

**STEP I - Ligand file format conversion**

Structures of semisynthetic derivative of curcumin were drawn using ChemSketch.

- Tools→clean structure.
- Tools→generate→SMILES notation.
- Copied the smile notation and uploaded the smiles in online smile translator-cactus.nci.nih.gov/services/translate.
- By choosing the required file format and save the file as pdb format.

**STEP II - Protein structure refinement**

The enzyme monoamine oxidase and indoleamine 2,3-dioxygenase was downloaded from RCSB (Research Co-laboratory for Structural Bioinformatics) Protein Data Bank and the protein was refined before use for docking.

- Opened Accelry's discovery studio viewer.
- File→open→RCSBPDB file.
- View→hierarchy→click water molecules→select all water molecules → delete.
- Selected ligand, which was unnecessary and deleted.
- Saved the molecule in a desired location.

**STEP III - Docking with Autodock 4.2**

- Open the refined protein from the location in pdb format.
- Preparation of target and ligand in AutoDock 4.

**STEP IV - Preparation of protein**

AutoDock 4.2 →File→Read molecule→Choose refined enzyme file.

Elimination of water molecule carried out by:

- Select→Select from string→Residue (*HOH*) →Add→Dismiss.
- Edit→Hydrogen→Add→Polar only→Ok.
- Edit→charges→Add kollmann charges→Ok.
- File→save→Write pdb→Browse→Save→Ok.
- Edit→Delete all molecules→Continue.

**STEP V – Docking**

1. Preparation of ligand

- Ligand→input→open.
- Ligand→torsion tree→detect root.
- Ligand→ torsion tree→show root expansion.
- Ligand→ torsion tree→choose torsions→done.

- Ligand→ torsion tree→set number of torsions→dismiss.
- Ligand→ torsion tree→hide root expansion.
- Ligand→ torsion tree→show/hide root marker.
- Ligand→output→ save as pdbqt file.
- Edit→delete→delete all molecules→ continue.

2. Conversion of pdb files of protein in to pdbqt file

- Grid→Macromolecule→Open→Save as pdbqt.

3. AutoGrid calculation and creating "gpf" file

- Grid→set map types→ open ligand.
- Grid→grid box→set 60 points in XYZ.
- File→close saving current.
- Grid→output→save as gpf.
- Edit→delete→delete all molecules→continue.

4. AutoDock calculation and creating 'dpf' file

- Docking→macromolecule→set rigid file name →open.
- Docking→ligand→open→accept.
- Docking→search parameters→genetic algorithm→accept.
- Docking→docking parameters→accept.
- Docking→output→lamarckian genetic algorithm→save as dpf

**Programming of 'Auto Grid' and 'Auto Dock' execution**

Open Cygwin64 and type as given below:

- cd c:
- cd cygwin64
- cd usr
- cd local
- cd bin

Program should list out the pdb, pdbqt, gpf and dpf files of an enzyme and ligand molecule. Then typed as: ./autogrid4.exe<space>–p<space>ligand.gpf<space> -l<space>ligand.glg If a ligand gets into the spacing of the grid, then the execution of this command will be '*Successful completion*'.

Then typed as: ./autodock4.exe<space>–p<space>ligand.dpf<space> -l<space>ligand.dlg

If the ligand binds to the amino acids through 10 different conformations, then the execution of this command will be; '*Successful completion*'.

**STEP VI - Viewing docking results**

1. Reading the docking log file. dlg

- Toggle the AutoDock Tools button.
- Analyse→Docking.
- Analyse→Conformations → Load.
- Double click on the conformation for to view it.

2. Visualizing docked conformations

- Analyse→Dockings→Play.
- Load dlg file.
- Choose the suitable conformations.

Analyse→Docking→Show Interactions.

3. Obtaining snap shots of docked pose

- File→Read Molecule.
- Analyse→Dockings→Open dlg file.
- Analyse→Macromolecule→Choose pdbqt file.
- Analyse→Conformations→Load.
- Double click the desired conformation.
- Analyse→Docking→Show Interactions.

Proteins and ligand interaction will be displayed. Zoom it and increase the contrast by holding right key and CTRL. Rapid energy evaluation was attained by pre-calculating the atomic resemblance potentials for each atom in the selected compounds. In the AutoGrid process, the target was enclosed on a three-dimensional grid point and the energy of interface of each atom in the compounds were encountered. The following docking factors were chosen for the Lamarckian genetic algorithm as follows: population size of 150 individuals, 2.5 million energy evaluations, maximum of 27000 generations, and number of top individuals to automatically survive to next generation of 1, mutation rate of 0.02, crossover rate of 0.8, 10 docking runs, and random initial positions and conformations. The probability of performing local search on a single compound in the population was set to 0.06. AutoDock was run various times to obtain various docked conformations, and used to calculate the predicted binding energy. Based on the binding energy results, two compounds showing highest and lowest binding energies will be selected and evaluated using *in vitro* studies. [75,76]

## SYNTHESIS OF ISATIN DERIVATIVES

### General scheme for preparation of synthetic derivative of Isatin

The synthesis of Isatin derivatives are shown in Fig.8.

### Synthesis of SDI 3

The ethanolic solution of Isatin (14.7g) was mixed with ethanolic solution of 4-amino benzoic acid (13.7g) and added 3-4 drops of glacial acetic acid. Then the mixture was refluxed for 2 hours at 60°C. The product obtained after the evaporation of solvent was filtered, washed with ethanol and recrystallised from ethanol. [77]

### Synthesis of SDI 7

The ethanolic solution of Isatin (14.7g) was mixed with ethanolic solution of 4-chloro aniline (12.7g) and added 3-4 drops of glacial acetic acid. Then the mixture was refluxed for 2 hours at 60°C. The product obtained after the evaporation of solvent was filtered, washed with ethanol and recrystallised from ethanol. [78]

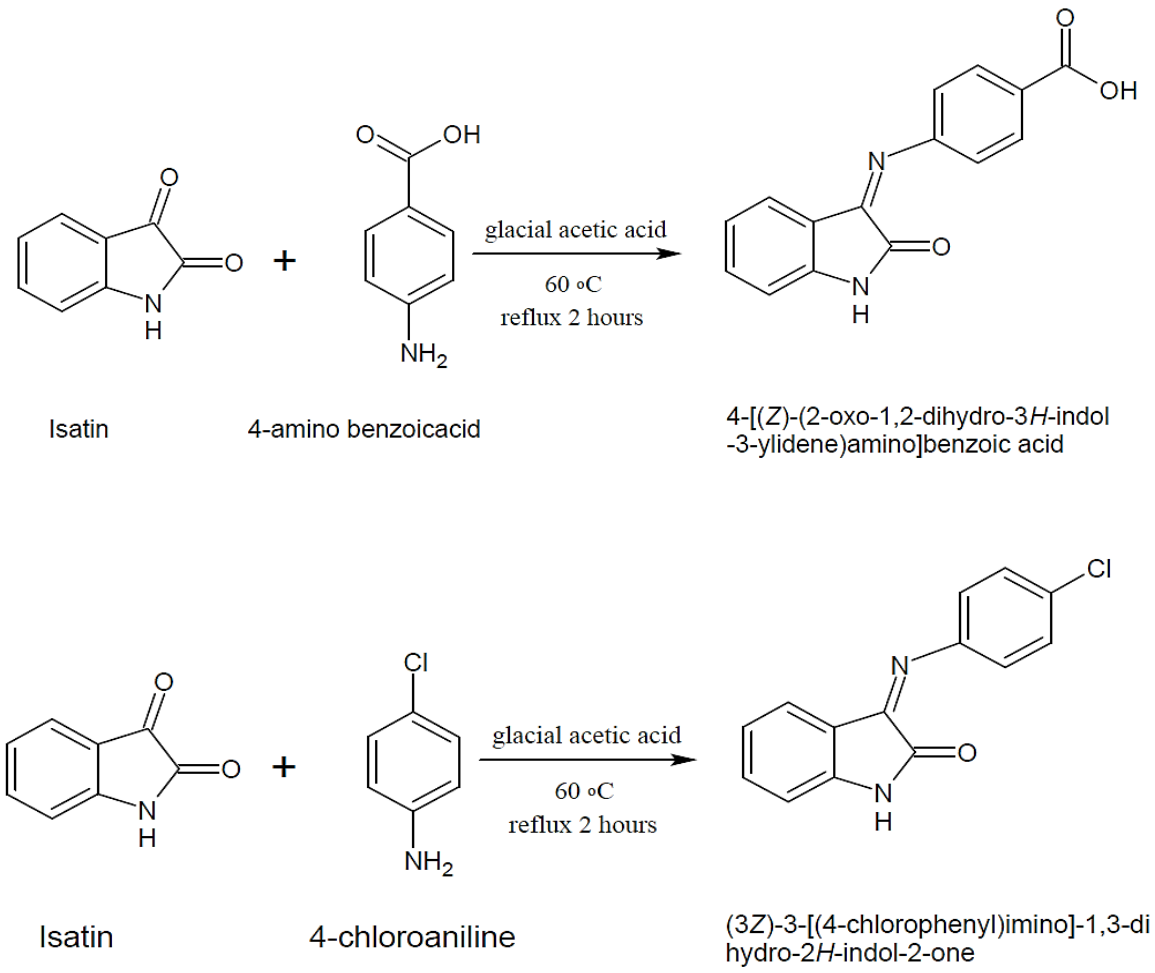



**Fig 8: Scheme used for preparation of SDI 3 and SDI 6**

## CHARACTERIZATION OF SDI

The newly synthesized compounds were characterized by checking their Rf value and melting points. The structures of the synthesized compounds were established through UV and IR spectroscopy.

### Determination of melting point

The melting point of the synthesized compounds will be determined by using open capillary tube MR-VIS visual melting range apparatus.

### Determination of $R_f$ value

The purity of the compound will be checked by TLC on silica gel G plates using ethyl acetate: n- hexane (1:2) solvent system and iodine vapours as visualizing agent. Chamber filled with vapour of iodine was used to detect the compounds eluted in the silica gel plate. [77,79]

### Confirmation by FTIR

In the ratio of 1:100, the isolated and purified isatin derivatives were mixed with infrared (IR) grade potassium bromide (KBr). The pellet of the mixture was prepared using a hydraulic press to apply 6 metric tons of pressure. In an inert environment, the pellet was scanned over a wave number range of 4000-400 cm-1 using FTIR equipment. [80]

## *IN-VITRO* MONOAMINE OXIDASE INHIBITOR ACTIVITY

### Experimental animals

Female Wistar rats weighing between 120 – 150 g were used for acute toxicity and male Wistar rats for antidepressant activity. The animals were procured from College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Mannuthi, Thrissur, Kerala.

### Brain homogenate preparation

Male *Wistar* rats weighing 150–250 g were sacrificed and the brains were removed. The entire brain minus cerebellum was homogenized in 9 volumes of ice-cold, phosphate-buffered 0.25 M sucrose, using a homogenizer. The homogenate was centrifuged at 1000 g for 10 min and the supernatant decanted and then recentrifuged at 18000 g for 20 min. The resulting pellet was resuspended in fresh 0.25 M sucrose and recentrifuged at 18000 g for 20 min. The washed pellet was resuspended in the original volume of 0.25 M sucrose and served as the tissue source for mitochondrial monoamine oxidase. [81]

**Estimation of MAO assay level**

The activities of MAO-A and MAO-B were determined by UV-Visible spectrophotometry. Dose dependent concentration of drug samples was added to 800 ml of solution containing potassium phosphate buffer (pH 7.60, 100 mM), 200 ml of MAO protein homogenates and benzylamine (for detecting MAO-B) or 5- HT (for detecting MAO-A) was incubated for 20 min at 37ºC. The reaction was started by adding 150 ml of 0.016 M benzylamine in 100 mM buffer or 200 ml of 0.02 M 5-HT, and incubated for 60 min. The reaction was stopped by adding 200 ml of 10% perchloric acid or 200 ml of 2M HCl. Then, 3ml of hexamethylene (for detecting MAO-B) or n-butyl acetate (for detecting MAO-A) was added to the reaction solution and the tubes were vortexed for 3 min. Following centrifugation at 10,625 g for 6 min, absorbance was determined using a spectrophotometer at 242 nm and 280 nm for MAO-B and MAO-A, respectively. [82]

***IN VIVO* STUDIES**

The study protocol was approved by the Institutional Animal Ethical Committee (COPSRIPMS/IAEC/PG/Pharmacology/001/2019-2020). The animals were maintained under controlled conditions of temperature (23±2˚C), humidity (50±5%) and 12-12h light- dark cycles. All the animals were acclimatized for 7 days before the study. The animals were randomized to experimental, normal and control groups, housed individually in sanitized polypropylene cages containing dust free paddy husk as bedding. They had free access to standard pellets as basal diet and drinking water ad libitum unless there will be restriction due to stress protocol. Animals were habituated to laboratory conditions for 48 h prior to experimental protocol to minimize non-specific stress if any.

**Acute toxicity and dose determination**

The acute toxicity study of the isatin derivative (SDI 3 and SDI 6) was performed based on fixed dose procedure employing the OECD guideline No. 420 (OECD 2002). [83]

**Procedure:**

The acute toxicity study was done by two steps – sighting study and main study. The purpose of the sighting study is to allow selection of the appropriate starting dose for the main study. Healthy adult female (generally slightly more sensitive than male) Wistar rats weighing between 120-150 g body weight was procured and kept in cages under ambient temperature (22 ± 3 $^{O}$C) with 12 hr light/dark cycle. The animals were randomly selected, marked and kept in their cages for 5 days prior to dosing for acclimatization to laboratory conditions. The animals were fasted over-night and were provided with water ad libitum. The synthesized test

compounds were suspended in 0.5% CMC and given orally. Totally 18 animals were used for this study. Sighting study was conducted for two compounds at dose levels 5, 50, 300 and 2000 mg/kg body weight (Table: 1). All animals survived without any toxic manifestations at doses of 5, 50, 300 and 2000 mg/kg during the sighting study and the maximum dose of 2000 mg/kg was selected for the main study. The main study was conducted for two compounds at dose of 2000 mg/kg body weight using 10 animals. After the administration of the compounds, food was withheld for further 3-4 hours.

Animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24 h (with special attention during the first 4 h) and daily thereafter for a period of 14 days. Once daily cage side observations included changes in skin and fur, eyes and mucous membrane (nasal), and also respiratory rate, circulatory (heart rate and blood pressure), autonomic (salivation, lacrimation, perspiration, piloerection, urinary incontinence and defecation) and central nervous system (drowsiness, gait, tremors and convulsions) changes. [83]

**Table 1: Design of acute toxicity studies**

| Study involved | Dose (mg/kg) | No. of animals |
|---|---|---|
| Sighting study | 5 | 1 |
| | 50 | 1 |
| | 300 | 1 |
| | 2000 | 1 |
| Main study | 2000 | 5 |

**Chronic unpredictable mild stress induced depression**

The animals were subjected to each stress once from day 1 to 7 respectively and this cycle was repeated for 6 periods (6 weeks). [84,85]

**Table 2: CUMS paradigm for the animals**

| Types of stressors used | Schedule of stressors in weeks/Days | | | | | |
|---|---|---|---|---|---|---|
| Food deprivation for 24 hours | 1 | 8 | 15 | 22 | 29 | 36 |
| Reverse light/dark cycle | 2 | 9 | 16 | 23 | 30 | 37 |
| Tail pinch for 2 min | 3 | 10 | 17 | 24 | 31 | 38 |
| Cage tilting (~45º inclined for 12h) | 4 | 11 | 18 | 25 | 32 | 39 |
| Wet soiled bedding for 24 hours | 5 | 12 | 19 | 26 | 33 | 40 |
| Cold swim at 15ºC for 5 minutes | 6 | 13 | 20 | 27 | 34 | 41 |
| Physical restraint for 10 minutes | 7 | 14 | 21 | 28 | 35 | 42 |

**Animal groups and drug treatment**

Around 42 male *Wistar* rats were divided into 7 groups (n=6 rats)

**Group I –** served as a control and received 0.5 % CMC, *p.o.* from 22$^{nd}$ to 42$^{nd}$ day.

**Group II** – served as negative control and received 0.5% CMC, *p.o.* from 22$^{nd}$ to 42$^{nd}$ day.

**Group III –**received SDI 3 at low dose *p.o.* from 22$^{nd}$ to 42$^{nd}$ day

**Group IV–** received SDI 3 at high dose *p.o.* from 22$^{nd}$ to 42$^{nd}$ day

**Group V**– received SDI 7 at low dose *p.o.* from 22$^{nd}$ to 42$^{nd}$ day

**Group VI–**received SDI 7 at high dose *p.o.* from 22$^{nd}$ to 42$^{nd}$ day

**Group VII-** received standard Selegiline at a dose of 15 mg/kg *p.o.* from 22$^{nd}$ to 42$^{nd}$ day.

Animals in group II to VII were exposed to CUMS from day 1 to day 42. During the period of study, behavioural assessment studies were performed once weekly for 6 weeks. The drug treatment was started after 3 weeks of CUMS exposure to all groups of rat. [84]

**BEHAVIOURAL ASSESSMENT**

**Table 3: Different behavioural parameters assessed for animals**

| Behavioural parameter | Schedule of behavioural assessment in weeks/Days | | | | | |
|---|---|---|---|---|---|---|
| Sucrose preference test | 2 | 9 | 16 | 23 | 30 | 37 |
| Elevated plus maze test | 3 | 10 | 17 | 24 | 31 | 38 |
| Forced swim test | 4 | 11 | 18 | 25 | 32 | 39 |
| Tail suspension test | 5 | 12 | 19 | 26 | 33 | 40 |
| Social interaction test | 6 | 13 | 22 | 27 | 34 | 41 |

**Sucrose preference test**

The sucrose preference test was carried out once weekly of 6-week CUMS exposure. The sucrose preference test was performed by housing the rats in individual cages with free access to two bottles containing 100 ml sucrose solution (1%, w/v) and 100 ml water. After 1 h, the volume of consumed sucrose solution and water were recorded and the sucrose preference was calculated by employing the following formula. [81,86]

$$\textbf{\textit{Sucrose preference}}\ (\%) = \frac{sucrose\ consumption}{(water\ consumption + sucrose\ consumption)} \times 100$$

**Forced swim test**

Thirty minutes after administration of test/standard drug, animals were individually allowed to swim inside a vertical Plexiglass cylinder (height: 40 cm; diameter: 18 cm, containing 15 cm of water maintained at 25 °C). Rats placed in the cylinders for the first time were initially highly active, vigorously swimming in circles, trying to climb the wall or diving to the bottom. After 2–3 min activity begins to subside and to be interspersed with phases of immobility or floating of increasing length. After 5–6 min, immobility reaches a plateau and the rats remain immobile for approximately 80% of the time. After 15 min in the water, the rats were removed and allowed to dry in a heated enclosure (32 °C) before being returned to their home cages. Floating behaviour during this 5 min period has been found to be reproducible in different groups of rats. An animal is judged to be immobile whenever it remains floating passively in the water in a slightly hunched but upright position, its nose just

above the surface. [81,87]

**Elevated plus-maze**

The plus-maze consists of two open arms, 50 × 10 ×40 cm, and two enclosed arms, 50 × 10 × 40 cm, with an open roof, arranged so that the two open arms are opposite to each other. The maze is elevated to a height of 50 cm. The rats were housed in pairs for 10 days prior to testing in the apparatus. During this time the rats were handled by the investigator on alternate days to reduce stress. Thirty minutes after oral administration of the test drug or the standard, the rat was placed in the centre of the maze, facing one of the enclosed arms. During a 5 min test period, time spent in the open and enclosed arms were observed. [81,88]

**Tail suspension test**

Animals were treated with the test compounds or the vehicle by oral administration 30 min prior to testing. For the test, the rats were suspended on the edge of a shelf 58 cm above a table top by adhesive tape placed approximately 1 cm from the tip of the tail. The duration of immobility is recorded for a period of 5 min. Rats are considered immobile when they hang passively and completely motionless for at least 1 min. [81]

**Social interaction test**

Animals are housed in groups of 6 animals. The apparatus used for detection of changes in social behavior and exploratory behavior consists of a perspex open topped box 51x51 cm and 20 cm height with 17x17 cm marked areas on the floor. One hour prior to the test, two animals from separate housing cages are treated with test compound orally They are placed into the box (with 60 W illumination 17 cm above) and their behaviour is observed over 10 min period by remote video recording. Social interaction between the animals is determined by timing the sniffing of partner, crawling under or climbing over the partner. [81]

**EUTHANASIA AND DISSECTION OF EXPERIMENTAL ANIMALS**

After 24 hours of last behavioural assessment, all the rats were anaesthetised under ketamine/xylazine anaesthesia, blood samples were collected by retro-orbital plexus puncture and subjected to centrifugation to isolate serum out of it. The serum was further used for estimation of various biochemical parameters. The animals were sacrificed by exsanguination, brain was excised and washed with ice cold saline solution. A 10% w/v tissue homogenate of prefrontal cortex and hippocampal region was prepared in ice-cold potassium phosphate buffer (100 mM, pH 7.4) and HCl-Butanol buffer followed by centrifugation at

4000 rpm for 10 min. The supernatant thus collected was used for the estimation of biochemical parameters. [84,89]

## HISTOPATHOLOGICAL STUDIES

At the end of study (43rd day), the prefrontal cortex and hippocampus region of the brain was excised and washed with ice cold saline. The tissue was fixed in 10% buffered neutral formalin solution. After fixation tissues were embedded in paraffin-wax and 5 µm thick sections were cut and stained with haematoxylin and eosin. The slides were observed under light microscope (10x), photomicrograph was taken and the histopathological changes were examined. [89]

## ESTIMATION OF BIOCHEMICAL PARAMETERS

## SERUM PARAMETERS

### Determination of Serum corticosterone

About 0.2 mL of serum and 2 mL of 0.1 mol/L NaOH were mixed for 30 s. To this 2 mL of dichloromethane was added into the mixture and scrolled for 3 min before standing for 5 min at the room temperature. After centrifugation at 2500 rpm for 20 min, 2 ml of sulfuric acid–alcohol (concentrated sulfuric acid: 98% ethanol = 7:3) mixed liquor was added into 1.5 mL organic phase. Next, the above solution was whirled for 3 min, centrifuged at 2500 rpm for 20 min, then the aqueous phase was allowed to stand for 30 min. Finally, the fluorescence intensity was measured at excitation wavelength of 472 nm and emission wavelength of 519 nm.[84]

### Estimation of kynurenine and tryptophan

The measurement of kynurenine/tryptophan ratio indirectly indicated indoleamine 2,3-dioxygenase (IDO) activity in the hippocampus. The serum isolated from the blood samples were stored at -20°C and the serum samples were thawed to room temperature during the time of estimation. About 500 µL of serum was mixed with 500 µL of 0.005 mol/L phosphate buffer (pH – 6.0) and the protein in the serum was precipitated by adding 125 µL of 2mM trichloroacetic acid. The mixture was then centrifuged at 13,000×g for 15 mins. The obtained supernatants were injected into column (5-µM, 12.5 cm of RP $C_{18}$ HPLC column). The mobile phase consisted of 0.015 mol/L phosphate buffer (pH – 6.4) and 2.7% acetonitrile. The sample was run at a flow rate of 2.0 ml/min. Kynurenine and tryptophan level was measured by UV absorbency at 360 and 280 nm. Standard calibration curve was plotted by simultaneously determining the linearity of kynurenine and tryptophan at a concentration

of 1mM/L. [90]

**TISSUE PARAMETERS**

**Estimation of serotonin and noradrenaline**

Weighed quantity of tissue (1.5 – 5 mg) and was homogenized in 0.1 mL HCl - butanol, (0.85 ml of 37% HCl in 1L n- butanol for spectroscopy) for 1 min in a cool environment. The sample was then centrifuged for 10 min at 2,000 rpm. About 0.08 mL of supernatant phase was removed and added to an Eppendorf reagent tube containing 0.2 mL of heptane (for spectroscopy) and 0.025 mL of 0.1 M hydrochloric acid. After 10 min of vigorous shaking, the tube was centrifuged under same conditions to separate two phases, aqueous phase (0.02 mL) was used for estimation of serotonin and nor adrenaline assay. All steps were carried out at 0 ºC. [89]

**Estimation of nor-adrenaline**

To 0.02ml of HCl phase, 0.05ml of 0.4 M HCl and 0.01ml EDTA were added, followed by 0.01ml iodine solution (0.1M in ethanol) for oxidation. The reaction mixture was allowed to stand for two minutes and after this addition of 0.01 ml $Na_2SO_3$ (Sodium sulphite), in 5 M NaOH (0.5 g $Na_2SO_3$ in 2 ml $H_2O$ + 18 ml 5 M NaOH) was added. Acetic acid (0.01 ml, 10 M) was added 1.5 minutes later. The resulting solution was then heated at 100ºC for 6 minutes. The solution is allowed to cool at room temperature and excitation and emission spectra were read in the microcuvette at excitation maxima. 395-485nm for NA. [89]

**Estimation of serotonin**

About 0.025ml of O-phthaldialdehyde (20 mg% in conc. HCl) were added to 0.02 ml of the HCl extract. The fluorophore was developed by heating at 100 °C for 10 min. After the samples reached equilibrium with the ambient temperature, excitation / emission spectra or intensity reading at 360-470 nm were taken in the micro cuvette. [89]

**Standard curve of norepinephrine hydrochloride**

The stock solution of norepinephrine hydrochloride was prepared at a concentration of 100 µg/ml in 0.1N HCl solution. Working standards were prepared in the range of 0.1 to 1.6 µg/ml by appropriate dilutions of the stock with 0.1N HCl. To 1ml of acetate buffer (0.02M acetic acid - pH 3) was added to each of the above dilutions. To the above solution 0.1ml of iodine reagent (1.27 g iodine is dissolved in 100 ml of absolute ethanol) was added. After 6 minutes the excess iodine was removed by addition of 0.2 ml of alkaline sulphite ($Na_2SO_3$ solution - 2.5 g of anhydrous salt in 10 ml of water) solution. The contents of the

tube were thoroughly mixed after each addition. Two minutes later 0.2 ml of 5 N acetic acid added and mixed well. All tubes were placed in a boiling water bath for 2 min, cooled in tap water and read for norepinephrine fluorescence. The solution was activated at 380 nm and the resulting fluorescence was measured at 480 nm in a spectrofluorimeter. The blank was a sample treated as above, except that the order of addition of iodine and thiosulphate reagents was reversed, i.e., omitting the step of oxidation. Standard curve of fluorescence vs. concentration was plotted. The fluorescence of norepinephrine extracted from the rat brain using solvent extraction method was extrapolated and concentration of norepinephrine from each rat brain was determined. The concentrations of norepinephrine were expressed as µg/g of the weight of rat brain. [91]

**Standard curve of 5-hydroxytryptamine (5-HT)**

The stock solution of 5-HT was prepared at a concentration of 10 µg / ml in 0.1N HCl. Working standards were prepared in the range of 0.1 to 0.5 µg/ml by appropriate dilutions of the stock with 0.1N HCl. 1.2 ml of O-Phthaldialdehyde were added and mixed well. All tubes were placed in a boiling water bath for 10 min, cooled in tap water. The above dilutions were directly measured with activation maxima at 355 nm and fluorescence maxima at 470 nm. Blank was measured using 0.1 N HCl and was subtracted from the standard absorbance. Standard curve of fluorescence vs. concentration was plotted. The fluorescence of 5-HT extracted from the rat brain using solvent extraction method was extrapolated and concentration of 5-HT from each rat brain was determined. The concentration of 5-HT was expressed as µg/g of the weight of rat brain. [91]

**Estimation of monoamine oxidase (MAO-A and MAO-B)**

Brain tissue suspended in 10 vol. of cold sodium phosphate buffer (10 mM, pH 7.4, containing 320 mM sucrose), was mingled at 4-8°C for 20 min. The mixture was centrifuged at 2500 rpm for 30 min and the pellets were re-suspended in the same buffer. The protein concentration was adjusted to 1 mg/ml. Protein concentration was estimated by the Lowry's method using bovine serum albumin as the standard. MAO activity was assessed spectrophotometrically. The assay mixtures contained 4 mM 5-HT as specific substrates for MAO-A and MAO-B, respectively. About 250 ml solution of the tissue homogenate, and 100 mM sodium phosphate buffer (pH 7.4) was taken up to a final volume of 1 ml. The reaction was allowed to proceed at 37°C for 20 min and stopped by adding 1 M HCl (200 ml), the reaction product was extracted with 5 ml of butyl acetate (for MAO-A assay) or cyclohexane (for MAO-B assay), respectively. The organic phase was measured at wavelength of 280 nm

for MAO-A assay and 242 nm for MAO-B assay with spectrophotometer, respectively. Blank samples were prepared by adding 1 M HCl (200 ml) prior to reaction, and worked up subsequently in the same manner. ($\varepsilon = 3\times10^4$) [92]

**Estimation of total protein content**

The amount of total protein in the tissue homogenate was estimated by the method of Lowry (1951) using bovine serum albumin as the standard. To 0.1mL of tissue homogenate, 4.0 mL of alkaline copper solution was added and allowed to stand for 10 min. Then, 0.4 ml of phenol reagent was added very rapidly, mixed quickly and incubated in room temperature for 30 min for colour development. Reading was taken against blank prepared with distilled water at 610 nm in UV-visible spectrophotometer. The protein content was calculated from standard curve prepared with bovine serum albumin and results were expressed as mmoles/min/mg wet tissue. [93]

**Assay of catalase (CAT)**

The assay of CAT was done by the method of Sinha, 1972. The reaction mixture contained 1.0 mL of 0.01 M phosphate buffer pH 7 and 0.1 mL of tissue homogenate and was incubated at 37$^{O}$C for 15 min. The reaction was started by the addition of 0.4 mL of $H_2O_2$. The reaction is stopped by the addition of 2.0 mL dichromate acetic acid reagent (5% potassium dichromate and glacial acetic acid are mixed in 1:3 ratio). The absorbance was measured at 620 nm. CAT activity was expressed as the amount of enzyme using the decomposition of μmoles $H_2O_2$/min/mg protein. The concentration of GSSH determined using molar coefficient value (ε) 43.6. [94]

**Assay of glutathione peroxidase (GPx)**

About 0.2 mL of the brain homogenate was mixed with 0.2 mL of 0.4 M Tris-buffer pH 7.0, 0.1 mL of 10 mM sodium azide, 0.1 mL of 0.042 % $H_2O_2$ and 0.2 mL of 200 mM glutathione and was incubated at 37 $^{O}$C for 10 min. The reaction was stopped by the addition of 0.1 mL 10% trichloroacetic acid and the absorbance was measured at 340 nm. GPx activity was expressed as nmoles/min/mg protein. The concentration of GPx determined using molar coefficient value (ε) $6.22\times10^{-3}$. [95]

**Assay of superoxide dismutase (SOD)**

The activity of SOD was determined according to the method of Kakkar, 1984. To 150 μL of brain homogenate, 1.8 mL of carbonate buffer (30 mM, pH 10.2), 0.7 mL of distilled water and 400 μL of epinephrine (45mM) were added and mixed well. The inhibition of autocatalyzed adrenochrome formation in the presence of brain tissue homogenate was

measured at 480 nm using a spectrophotometer. Autooxidation of epinephrine to adrenochrome was performed in a control tube without the homogenate. SOD activity was expressed as nmoles/min/mg protein. The concentration of SOD determined using molar coefficient value (ε) 4020. [96]

**Assay of glutathione reductase (GSSH)**

The activity of GSSH in the tissue was determined by the method of Racker, 1955. The reaction mixture contained 2.1 mL of 0.25 mM potassium phosphate buffer pH 7.6, 0.1 mL of 0.001 M NADPH, 0.2 mL of 0.0165 M oxidized glutathione and 0.1 mL of bovine serum albumin (10 mg/mL). The reaction was started by the addition of 0.02 mL of tissue homogenate. The absorbance was measured at 340 nm against a blank. GSSH activity was expressed as nmoles/min/mg protein. The concentration of GSSH determined using molar coefficient value (ε) $6.22\times10^{-3}$. [97]

**Assay of reduced glutathione (GSH)**

The activity of GSH was determined by Ellman's method. About 1.0 mL of tissue homogenate was treated with 0.5 mL of Ellman's reagent (19.8 mg of 5,5'- Dithiobis-(2-Nitro benzoic acid) [DTNB] in 100 mL of 0.1 % sodium citrate) and 3.0 mL of phosphate buffer (0.2 M, pH-8). The absorbance was read at 412 nm using a spectrophotometer. GSH activity was expressed as nmoles/min/mg protein. The concentration of GSSH determined using molar coefficient value (ε) 13600. [98]

**Assay of malondialdehyde (MDA)**

The level of lipid peroxidation was measured as malondialdehyde (MDA) according to the method of Niehaus and Samuelson, (1986). About 0.1 mL of the tissue homogenate was combined with 2 mL of TCA-TBA-HCl reagent (1:1:1) (15% trichloro acetic acid (TCA) and 0.375% thiobarbituric acid (TBA) in 0.25 N HCl) and placed in water bath for 15 min, cooled and centrifuged at 100xg for 10 min. The precipitate was removed after cooling by centrifugation at 1000xg for 10 min. The absorbance of clear supernatant was measured against a reference blank at 535 nm. The levels of MDA were calculated using extinction coefficient calculation. The values are expressed as nmoles of MDA formed/min/mg protein. [99,100]

**STATISTICAL ANALYSIS**

Statistical analysis was performed using one-way ANOVA followed by Dunnett's test using the software GraphPad Instat, USA. The values were expressed as a mean ± standard error of the mean (SEM) and $P< 0.05$ was considered statistically significant.

## RESULTS

## *IN SILICO* STUDIES

### Evaluation of drug likeness properties of the SDI compounds

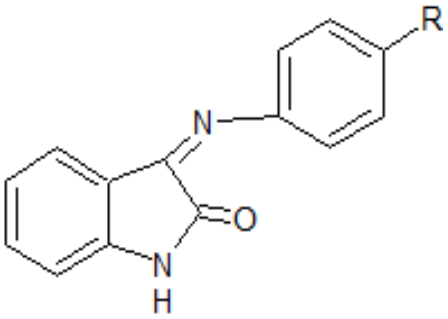
*In silico* predictions of the drug likeness properties are useful in the drug development process of a new chemical entity. The drug likeness property of a compound was evaluated based on the Lipinski's Rule of Five (Ro5). The parameters such as molecular weight, log P, number of violations, hydrogen bond donors and hydrogen bond acceptors of the compound were calculated for the Ro5. The compounds with no violation were considered for further *in silico* screening studies.

The possible structural fragments of the synthetic derivatives of isatin were designed (Table 4) with the help of ChemSketch and they were subjected to test their drug likeness properties and bioactivity scores with the help of Molinspiration server. The 50 different compounds of SDI, i.e., SDI 1 to SDI 50 were designed and evaluated for Lipinski rule of Five. The calculated values of various parameters of the SDI for drug likeness were calculated and tabulated.

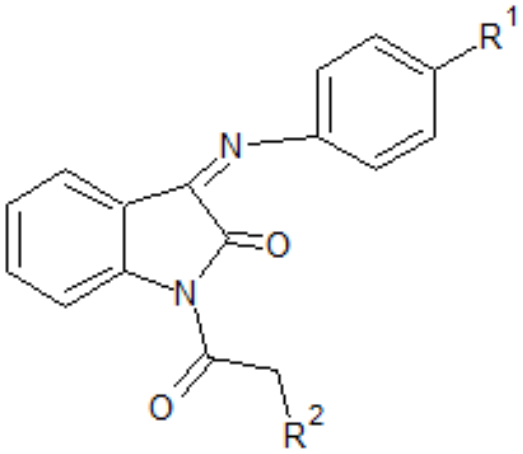
**Rule of five (Ro5) analysis**:

Lipinski's rule is widely used to determine molecular properties that are important for drug's pharmacokinetic *in vivo*. According to Lipinski's rule of five (Ro5), a candidate molecule is more likely to be orally active if: a) the molecular weight is below 500, b) the calculated octanol/water partition coefficient (log P) is less than 5, c) there are not more than 5 hydrogen bond donors (OH and NH groups), d) there are not more than 10 hydrogen bond acceptors (notably N and O). The Lipinski parameters of the SDI are tabulated in Table 5. It was found that among the 50 different SDIs used for calculations of molecular properties. In that 10 compounds (SDI 13, SDI 14, SDI 20, SDI 43, SDI 44, SDI 46-SDI 50) show violation and moreover out of 10 compounds, SDI 47 shows 2 violations, the remaining 9 compounds shows 1 violation. Compounds showing 2 or more violations were not acceptable for the further developmental studies, so these compounds were rejected from further *in silico* studies. The remaining 40 compounds had exhibited zero violation and were selected for the further *in silico* study.

**Table 4: List of synthetic derivatives of isatin selected for *in silico* study**

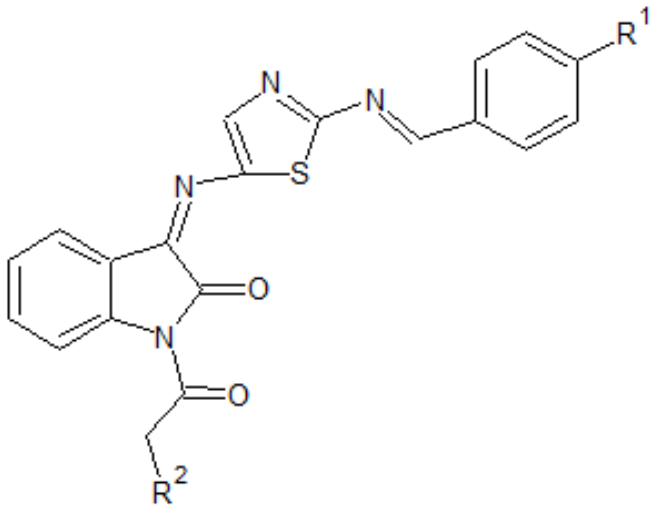



| S.No. | Compound Code | R |
|---|---|---|
| 1 | SDI 1 | $-CH_3$ |
| 2 | SDI 2 | $-C_2H_5$ |
| 3 | SDI 3 | -COOH |
| 4 | SDI 4 | -Br |
| 5 | SDI 5 | $-NO_2$ |
| 6 | SDI 6 | -Cl |




| S.No. | Compound Code | $R^1$ | $R^2$ |
|---|---|---|---|
| 7 | SDI 7 | -Cl | $-NC_4H_8$ |
| 8 | SDI 8 | -Cl | $-NC_5H_{10}$ |
| 9 | SDI 9 | -Cl | $-NC_4H_8O$ |
| 10 | SDI 10 | -Cl | $-N_2C_5H_{11}$ |
| 11 | SDI 11 | -Cl | $-NC_2H_6$ |

| | | | |
|---|---|---|---|
| 12 | SDI 12 | -Cl | $-NC_4H_{10}$ |
| 13 | SDI 13 | -Cl | $-NC_6H_{14}$ |
| 14 | SDI 14 | -Cl | $-NC_6H_{14}$ |
| 15 | SDI 15 | -Br | $-NC_4H_4$ |
| 16 | SDI 16 | $-NC_4H_8$ | $-NC_2H_6$ |
| 17 | SDI 17 | $-NC_5H_{10}$ | $-NC_4H_{10}$ |
| 18 | SDI 18 | $-NC_5H_{10}$ | $-NC_6H_{14}$ |
| 19 | SDI 19 | $-N_2C_5H_{11}$ | $-NC_6H_{14}$ |
| 20 | SDI 20 | $-NC_6H_{14}$ | -NC4H8 |
| 21 | SDI 21 | $-NC_4H_{10}$ | -NC5H10 |
| 22 | SDI 22 | $-NC_2H_6$ | -NC4H8O |
| 23 | SDI 23 | $-NC_2H_6$ | $-N_2C_5H_{11}$ |
| 24 | SDI 24 | $-NC_4H_8$ | -Cl |
| 25 | SDI 25 | $-NC_5H_{10}$ | -COOH |
| 26 | SDI 26 | $-NC_4H_6O$ | $-NO_2$ |
| 27 | SDI 27 | $-N_2C_5H_{11}$ | -Br |
| 28 | SDI 28 | $-NC_4H_6O$ | -COOH |
| 29 | SDI 29 | - $NC_4H_8$ | - $NC_4H_8$ |
| 30 | SDI 30 | -Br | -COOH |



| S.No. | Compound Code | $R^1$ | $R^2$ |
|---|---|---|---|
| 31 | SDI 31 | -H | $-CH_3$ |
| 32 | SDI 32 | -H | -Cl |
| 33 | SDI 33 | -H | -COOH |
| 34 | SDI 34 | -H | $-NO_2$ |
| 35 | SDI 35 | -H | $-C_2H_5$ |
| 36 | SDI 36 | -H | $-NC_4H_8$ |
| 37 | SDI 37 | -H | $-NC_5H_{10}$ |
| 38 | SDI 38 | -H | $-NC_4H_8O$ |
| 39 | SDI 39 | -H | $-N_2C_5H_{11}$ |
| 40 | SDI 40 | -H | $-NC_4H_6O$ |
| 41 | SDI 41 | -H | $-NC_2H_6$ |
| 42 | SDI 42 | -H | $-NC_4H_{10}$ |
| 43 | SDI 43 | -H | $-NC_6H_{14}$ |
| 44 | SDI 44 | -H | $-NC_6H_{14}$ |
| 45 | SDI 45 | -Cl | $-NC_2H_6$ |
| 46 | SDI 46 | $-CH_3$ | $-NC_4H_{10}$ |
| 47 | SDI 47 | -COOH | $-NC_6H_{14}$ |
| 48 | SDI 48 | $-NC_4H_8O$ | $-NC_2H_6$ |
| 49 | SDI 49 | $-N_2C_5H_{11}$ | $-NC_4H_8$ |
| 50 | SDI 50 | $-NC_4H_8$ | -Cl |

Molinspiration was also used to predict the bioactivity scores of each derivative. The predicted bioactivity scores of screened compounds for GPCR ligand, ion channel modulator, kinase inhibitor, nuclear receptor ligand, protease inhibitor and enzyme inhibition were studied and tabulated in Table 6. As a general rule, larger is the bioactivity score, higher is the probability that investigated compound will be active. Therefore, a molecule having bioactivity score more than 0.00 is most likely to possess considerable biological activities, while values -0.50 to 0.00 are expected to be moderately active and if score is less than -0.50 it is presumed to be inactive. The drug likeliness scores as calculated through Molinspiration reveals that the selected compounds satisfy maximum parameters.

The predicted bioactivities of the compounds of SDI were tabulated in Table 6. Considerable to moderate GPCR ligand action were observed with the SDI compounds within a range of -0.01 to -0.71. Predicted ion channel modulatory effects was within the range -0.56 to -1.10. Kinase inhibitory score was found to be within a range between 0.01 to -0.72. The predicted nuclear receptor ligand activity was within a range of -0.49 to -1.74. The protease inhibition score was found to be within a range of -0.53 to -1.42 and indicate their considerable to inactive action. The enzyme inhibitory prediction was within a range of -0.08 to -0.56.

The compounds which showed more than 1 violation were rejected as they might not have drug likeliness property. The compounds showed 0 or 1 violation were selected and used for further *in silico* studies.

**Table 5: Calculation of molecular properties of SDI 1 to SDI 50 and standard Selegiline using Molinspiration online tool**

| S. No | Compound code | Log P | Molecular weight | nON | nOHNH | Violation |
|---|---|---|---|---|---|---|
| 1 | SDI 1 | 3.69 | 236.27 | 3 | 1 | 0 |
| 2 | SDI 2 | 4.15 | 250.30 | 3 | 1 | 0 |
| 3 | **SDI 3** | **3.15** | **266.26** | **5** | **2** | **0** |
| 4 | SDI 4 | 4.05 | 301.14 | 3 | 1 | 0 |
| 5 | SDI 5 | 3.20 | 267.24 | 6 | 1 | 0 |
| 6 | **SDI 6** | **3.92** | **256.69** | **3** | **1** | **0** |
| 7 | SDI 7 | 4.29 | 367.84 | 5 | 0 | 0 |
| 8 | SDI 8 | -0.92 | 376.82 | 5 | 0 | 0 |
| 9 | SDI 9 | -2.96 | 379.80 | 6 | 0 | 0 |
| 10 | SDI 10 | -3.56 | 392.85 | 6 | 0 | 0 |
| 11 | SDI 11 | 3.89 | 341.80 | 5 | 0 | 0 |
| 12 | SDI 12 | 4.64 | 369.85 | 5 | 0 | 0 |
| 13 | SDI 13 | 5.65 | 397.91 | 5 | 0 | 1 |
| 14 | SDI 14 | 5.24 | 397.91 | 5 | 0 | 1 |
| 15 | SDI 15 | 4.43 | 412.29 | 5 | 0 | 0 |
| 16 | SDI 16 | 3.72 | 376.46 | 6 | 0 | 0 |
| 17 | SDI 17 | 1.19 | 413.50 | 6 | 0 | 0 |
| 18 | SDI 18 | 1.78 | 441.56 | 6 | 0 | 0 |
| 19 | SDI 19 | -2.38 | 457.58 | 7 | 0 | 0 |
| 20 | SDI 20 | 5.07 | 432.57 | 6 | 0 | 1 |
| 21 | SDI 21 | -0.71 | 413.50 | 6 | 0 | 0 |
| 22 | SDI 22 | -3.54 | 388.43 | 7 | 0 | 0 |
| 23 | SDI 23 | -4.13 | 401.47 | 7 | 0 | 0 |
| 24 | SDI 24 | 0.28 | 220.26 | 5 | 4 | 0 |
| 25 | SDI 25 | 1.90 | 244.28 | 5 | 3 | 0 |

| S. No | Compound code | Log P | Molecular weight | nON | nOHNH | Violation |
|---|---|---|---|---|---|---|
| 26 | SDI 26 | 4.29 | 367.84 | 5 | 0 | 0 |
| 27 | SDI 27 | 3.71 | 391.43 | 7 | 1 | 0 |
| 28 | SDI 28 | 2.92 | 394.39 | 9 | 0 | 0 |
| 29 | SDI 29 | 3.92 | 441.33 | 6 | 0 | 0 |
| 30 | SDI 30 | 2.06 | 391.38 | 8 | 1 | 0 |
| 31 | SDI 31 | 4.40 | 346.42 | 5 | 1 | 0 |
| 32 | SDI 32 | 4.63 | 366.83 | 5 | 1 | 0 |
| 33 | SDI 33 | 3.86 | 376.40 | 7 | 2 | 0 |
| 34 | SDI 34 | 3.91 | 377.38 | 8 | 1 | 0 |
| 35 | SDI 35 | 4.86 | 360.44 | 5 | 1 | 0 |
| 36 | SDI 36 | 4.45 | 401.50 | 6 | 1 | 0 |
| 37 | SDI 37 | 4.96 | 415.52 | 6 | 1 | 0 |
| 38 | SDI 38 | 3.90 | 417.49 | 7 | 1 | 0 |
| 39 | SDI 39 | 3.94 | 430.54 | 7 | 1 | 0 |
| 40 | SDI 40 | 3.31 | 415.48 | 7 | 1 | 0 |
| 41 | SDI 41 | 4.05 | 375.46 | 6 | 1 | 0 |
| 42 | SDI 42 | 4.80 | 403.51 | 6 | 1 | 0 |
| 43 | SDI 43 | 5.40 | 431.56 | 6 | 1 | 1 |
| 44 | SDI 44 | 5.81 | 431.56 | 6 | 1 | 1 |
| 45 | SDI 45 | 4.60 | 451.94 | 7 | 0 | 0 |
| 46 | SDI 46 | 5.13 | 459.57 | 7 | 0 | 1 |
| 47 | SDI 47 | 5.18 | 517.61 | 9 | 1 | 2 |
| 48 | SDI 48 | 3.87 | 502.60 | 9 | 0 | 1 |
| 49 | SDI 49 | 4.32 | 541.68 | 9 | 0 | 1 |
| 50 | SDI 50 | 5.01 | 477.98 | 7 | 0 | 1 |
| 51 | Selegiline | 2.64 | 187.29 | 1 | 0 | 0 |

**Table 6: Predicted bioactivity score for SDI 1 to SDI 50 and standard Selegiline using Molinspiration online tool**

| S. No | Compound code | GPCR ligand | Ion channel modulator | Kinase inhibitor | Nuclear receptor ligand | Protease inhibitor | Enzyme inhibitor |
|---|---|---|---|---|---|---|---|
| 1 | SDI 1 | -0.55 | -0.85 | -0.48 | -1.23 | -1.39 | -0.47 |
| 2 | SDI 2 | -0.40 | -0.70 | -0.43 | -1.02 | -1.17 | -0.34 |
| 3 | **SDI 3** | **-0.34** | **-0.69** | **-0.35** | **-0.78** | **-1.04** | **-0.24** |
| 4 | SDI 4 | -0.63 | -0.85 | -0.47 | -1.34 | -1.47 | -0.48 |
| 5 | SDI 5 | -0.54 | -0.72 | -0.47 | -1.08 | -1.25 | -0.43 |
| 6 | **SDI 6** | **-0.50** | **-0.75** | **-0.44** | **-1.22** | **-1.37** | **-0.43** |
| 7 | SDI 7 | -0.01 | -0.97 | -0.39 | -0.81 | -0.81 | -0.41 |
| 8 | SDI 8 | -0.10 | -0.62 | -0.50 | -1.40 | -0.80 | -0.26 |
| 9 | SDI 9 | -0.21 | -0.78 | -0.51 | -1.29 | -0.88 | -0.37 |
| 10 | SDI 10 | -0.10 | -0.61 | -0.44 | -1.38 | -0.73 | -0.22 |
| 11 | SDI 11 | -0.08 | -1.09 | -0.43 | -0.96 | -1.01 | -0.48 |
| 12 | SDI 12 | -0.08 | -1.03 | -0.45 | -0.88 | -0.94 | -0.47 |
| 13 | SDI 13 | -0.02 | -0.93 | -0.42 | -0.78 | -0.82 | -0.41 |
| 14 | SDI 14 | -0.05 | -0.92 | -0.45 | -0.81 | -0.80 | -0.45 |
| 15 | SDI 15 | -0.11 | -1.04 | -0.41 | -0.90 | -0.88 | -0.45 |
| 16 | SDI 16 | -0.04 | -0.92 | -0.33 | -0.81 | -0.81 | -0.39 |
| 17 | SDI 17 | -0.33 | -0.69 | -0.72 | -1.28 | -0.76 | -0.19 |
| 18 | SDI 18 | -0.28 | -0.61 | -0.71 | -1.20 | -0.65 | -0.19 |
| 19 | SDI 19 | -0.19 | -0.56 | -0.62 | -1.28 | -0.62 | -0.08 |
| 20 | SDI 20 | 0.08 | -0.73 | -0.31 | -0.63 | -0.53 | -0.35 |
| 21 | SDI 21 | -0.07 | -0.56 | -0.43 | -1.20 | -0.70 | -0.24 |
| 22 | SDI 22 | -0.19 | -0.74 | -0.44 | -1.16 | -0.81 | -0.32 |
| 23 | SDI 23 | -0.08 | -0.57 | -0.37 | -1.25 | -0.66 | -0.18 |
| 24 | SDI 24 | -0.25 | -1.03 | -0.65 | -1.74 | -1.42 | -0.56 |
| 25 | SDI 25 | -0.61 | -0.62 | 0.09 | -1.76 | -1.27 | -0.29 |

| S. No | Compound code | GPCR ligand | Ion channel modulator | Kinase inhibitor | Nuclear receptor ligand | Protease inhibitor | Enzyme inhibitor |
|---|---|---|---|---|---|---|---|
| 26 | SDI 26 | -0.27 | -1.00 | -0.31 | -0.79 | -0.82 | -0.49 |
| 27 | SDI 27 | -0.02 | -0.82 | -0.30 | -0.49 | -0.59 | -0.28 |
| 28 | SDI 28 | -0.28 | -0.98 | -0.39 | -0.80 | -0.83 | -0.41 |
| 29 | SDI 29 | -0.08 | -0.83 | -0.21 | -0.70 | -0.76 | -0.38 |
| 30 | SDI 30 | -0.14 | -0.98 | -0.43 | -0.77 | -0.61 | -0.42 |
| 31 | SDI 31 | -0.65 | -0.92 | -0.10 | -1.14 | -1.06 | -0.36 |
| 32 | SDI 32 | -0.61 | -0.85 | -0.08 | -1.13 | -1.05 | -0.36 |
| 33 | SDI 33 | -0.55 | -0.80 | -0.10 | -0.90 | -0.90 | -0.25 |
| 34 | SDI 34 | -0.71 | -0.84 | -0.19 | -1.13 | -1.07 | -0.41 |
| 35 | SDI 35 | -0.57 | -0.81 | -0.12 | -1.04 | -0.96 | -0.31 |
| 36 | SDI 36 | -0.49 | -0.72 | -0.02 | -0.98 | -0.85 | -0.30 |
| 37 | SDI 37 | -0.45 | -0.69 | -0.03 | -0.92 | -0.82 | -0.28 |
| 38 | SDI 38 | -0.51 | -0.76 | 0.01 | -0.97 | -0.86 | -0.31 |
| 39 | SDI 39 | -0.39 | -0.64 | -0.05 | -0.96 | -0.82 | -0.29 |
| 40 | SDI 40 | -0.57 | -0.85 | -0.17 | -1.19 | -0.83 | -0.42 |
| 41 | SDI 41 | -0.56 | -0.81 | -0.03 | -1.02 | -0.96 | -0.32 |
| 42 | SDI 42 | -0.51 | -0.77 | -0.05 | -0.97 | -0.92 | -0.34 |
| 43 | SDI 43 | -0.41 | -0.64 | -0.04 | -0.90 | -0.72 | -0.32 |
| 44 | SDI 44 | -0.43 | -0.69 | -0.05 | -0.89 | -0.81 | -0.30 |
| 45 | SDI 45 | -0.39 | -1.10 | -0.27 | -1.09 | -0.99 | -0.45 |
| 46 | SDI 46 | -0.39 | -1.10 | -0.30 | -1.02 | -0.95 | -0.45 |
| 47 | SDI 47 | -0.29 | -0.97 | -0.29 | -0.78 | -0.74 | -0.33 |
| 48 | SDI 48 | -0.34 | -1.03 | -0.17 | -0.95 | -0.84 | -0.39 |
| 49 | SDI 49 | -0.20 | -0.98 | -0.15 | -0.87 | -0.71 | -0.37 |
| 50 | SDI 50 | -0.53 | -1.04 | -0.17 | -0.95 | -0.86 | -0.47 |
| 51 | Selegiline | -0.05 | -0.06 | -0.61 | -0.66 | -0.35 | 0.04 |

**ADMET studies**

ADMET studies were carried out using *Pre*ADMET software for the SDI compounds and the results were tabulated in Table 7. Numerous *in vitro* methods have been used in the drug selection process for assessing the intestinal absorption of drug candidates. Among them, Caco2-cell model and MDCK (Madin-Darby Canine Kidney) cell model has been recommended as a reliable *in vitro* model for the prediction of oral drug absorption. Additionally, *in silico* HIA (human intestinal absorption) model and skin permeability model can predict and identify potential drug for oral delivery and transdermal delivery. In distribution, BBB (blood brain barrier) penetration can give information of therapeutic drug in the central nervous system (CNS), plasma protein binding model in its disposition and efficacy.

Chemicals which are strongly bound will have more than 90% of protein binding and chemicals weakly bound will have less than 90% of protein binding. BBB value more than 1.0 shows high absorption to CNS and 1.0 ~ 0.1 shows moderate absorption to CNS and less than 0.1 shows low absorption. HIA values between 0 ~ 20 % shows poorly absorbed compounds and values between 20 ~ 70 % shows moderately absorbed compounds, between 70 ~ 100 % shows well absorbed compounds. For prediction of Caco-2 cell permeability in *Pre*ADMET, chemical structures at pH 7.4 are applied, because Caco-2 cell permeability and MDCK cell permeability are measured at about pH 7.4. Caco-2 cells are derived from human colon adenocarcinoma and possess multiple drug transport pathways through intestinal epithelium. Values less than 4 shows low permeability and between 4 ~ 70 shows moderate permeability. Caco-2 value between more than 70 shows high permeability.

The SDI compounds which exhibited blood brain barrier penetration more the 1.0 were only selected and used for further *in silico* studies. On account of that the SDI compounds were evaluated for ADMET properties. The table shows that, SDI 3, SDI 6, SDI 16, SDI 26, SDI 43, SDI 46 shows BBB penetration more than 1.0. Hence, these compounds were selected further for *in silico* docking studies. The selected SDI compounds also exhibited optimum ADMET values. The leads were found to have good oral absorption, intestinal absorption, solubility and less interaction.

**Table 7: Calculated ADMET properties of selected SDI using *PreADMET* online tool**

| S. NO | Compound code | Blood brain barrier | Buffer solubility mg/L | Caco2 | CYP 3A4 inhibition | CYP 3A4 substrate | HIA | MDCK | Plasma Protein Binding |
|---|---|---|---|---|---|---|---|---|---|
| 1 | SDI 1 | 0.0965927 | 131.046 | 26.8692 | Inhibitor | Non | 96.127205 | 285.411 | 87.725840 |
| 2 | SDI 2 | 0.204348 | 75.3164 | 30.8737 | Inhibitor | Non | 96.201008 | 208.5 | 88.928257 |
| 3 | **SDI 3** | **1.004631** | **1436.74** | **19.362** | **Non** | **Non** | **97.154414** | **70.0825** | **87.543376** |
| 4 | SDI 4 | 0.940859 | 55.0036 | 24.0716 | Inhibitor | Non | 96.700115 | 8.71679 | 87.021385 |
| 5 | SDI 5 | 0.93593 | 20281.1 | 9.80894 | Non | Non | 84.457079 | 35.654 | 87.991329 |
| 6 | **SDI 6** | **1.02672** | **113.178** | **25.6941** | **Non** | **Non** | **96.429570** | **244.808** | **89.750838** |
| 7 | SDI 7 | 0.859704 | 34.4836 | 35.4608 | Non | Substrate | 97.404491 | 121.951 | 82.733321 |
| 8 | SDI 8 | 0.617743 | 17.6183 | 38.47 | Non | Substrate | 97.438739 | 115.061 | 84.919800 |
| 9 | SDI 9 | 0.367728 | 125.526 | 35.8626 | Non | Substrate | 97.465791 | 64.5757 | 77.899772 |
| 10 | SDI 10 | 0.0492912 | 293.89 | 41.626 | Non | Weakly | 97.430101 | 69.3593 | 79.980869 |
| 11 | SDI 11 | 0.612112 | 128.857 | 35.9513 | Non | Substrate | 97.367158 | 148.896 | 77.820473 |
| 12 | SDI 12 | 0.811214 | 31.904 | 42.5612 | Non | Substrate | 97.404491 | 115.28 | 83.770863 |
| 13 | SDI 13 | 0.3187 | 7.32146 | 47.4387 | Non | Substrate | 97.480317 | 83.7169 | 84.465791 |
| 14 | SDI 14 | 0.656966 | 7.85376 | 44.8747 | Non | Substrate | 97.480317 | 64.9058 | 84.620590 |
| 15 | SDI 15 | 0.850103 | 16.0113 | 34.6195 | Non | Substrate | 97.542260 | 3.77408 | 82.161037 |
| 16 | SDI 16 | 1.02957 | 21.5226 | 46.7299 | Non | Substrate | 97.391784 | 8.12894 | 76.646070 |
| 17 | SDI 17 | 0.984096 | 2.6946 | 54.3683 | Non | Substrate | 97.414512 | 36.358 | 87.922848 |
| 18 | SDI 18 | 0.607919 | 0.657885 | 55.9351 | Non | Substrate | 97.485482 | 3.55892 | 88.476907 |
| 19 | SDI 19 | 0.364984 | 2.74544 | 56.1369 | Non | Weakly | 97.470992 | 16.8062 | 48.453741 |
| 20 | SDI 20 | 0.977131 | 1.29476 | 55.7399 | Non | Substrate | 97.446008 | 0.0708955 | 87.097864 |
| 21 | SDI 21 | 0.815265 | 2.6946 | 54.3306 | Non | Substrate | 97.414512 | 42.0228 | 88.455210 |
| 22 | SDI 22 | 0.364333 | 78.2711 | 48.8929 | Non | Substrate | 97.719939 | 108.789 | 61.947579 |
| 23 | SDI 23 | 0.221238 | 44.7993 | 55.5823 | Non | Weakly | 97.423876 | 8.99892 | 80.710444 |
| 24 | SDI 24 | 0.409624 | 6183.62 | 20.6377 | Non | Non | 92.942771 | 138.166 | 44.701803 |
| 25 | SDI 25 | 0.152766 | 192.221 | 4.20821 | Non | Non | 93.786182 | 66.243 | 35.102192 |

| S. NO | Compound code | Blood brain barrier | Buffer solubility mg/L | Caco2 | CYP 3A4 inhibition | CYP 3A4 substrate | HIA | MDCK | Plasma Protein Binding |
|---|---|---|---|---|---|---|---|---|---|
| 26 | SDI 26 | 1.11896 | 5.38359 | 22.171 | Non | Substrate | 97.428527 | 0.358892 | 93.496080 |
| 27 | SDI 27 | 0.718756 | 3352.04 | 21.0392 | Non | Non | 99.481765 | 0.169679 | 94.486203 |
| 28 | SDI 28 | 0.13616 | 17382 | 8.64577 | Non | Weakly | 90.900074 | 1.25233 | 93.388426 |
| 29 | SDI 29 | 0.504007 | 1.55934 | 22.5927 | Non | Weakly | 97.583652 | 0.0263926 | 81.784134 |
| 30 | SDI 30 | 0.118989 | 51610.9 | 20.6812 | Non | Non | 98.306835 | 0.364565 | 92.038565 |
| 31 | SDI 31 | 0.364761 | 0.0812915 | 32.0924 | Non | Weakly | 97.748520 | 34.396 | 100.0 |
| 32 | SDI 32 | 0.659465 | 0.0684318 | 45.9177 | Non | Weakly | 97.280760 | 23.7733 | 100.0 |
| 33 | SDI 33 | 0.105826 | 0.859343 | 2.29929 | Non | Non | 96.558480 | 0.117707 | 98.994689 |
| 34 | SDI 34 | 0.139931 | 12.1041 | 0.677568 | Non | Weakly | 88.715589 | 0.0747344 | 100.0 |
| 35 | SDI 35 | 0.442503 | 0.0458885 | 32.9136 | Non | Weakly | 97.614472 | 20.6726 | 100.0 |
| 36 | SDI 36 | 0.666825 | 0.0113582 | 45.964 | Non | Weakly | 97.468129 | 5.35033 | 100.0 |
| 37 | SDI 37 | 0.799513 | 0.00578527 | 45.475 | Non | Weakly | 97.353632 | 13.2758 | 100.0 |
| 38 | SDI 38 | 0.258933 | 0.0412012 | 43.3878 | Non | Substrate | 98.179306 | 18.6942 | 100.0 |
| 39 | SDI 39 | 0.271397 | 0.0259609 | 46.0759 | Non | Weakly | 97.566745 | 17.7279 | 79.597480 |
| 40 | SDI 40 | 0.2391 | 0.0890755 | 37.9686 | Non | Weakly | 98.670112 | 0.355245 | 100.0 |
| 41 | SDI 41 | 0.505642 | 0.0427139 | 44.5629 | Non | Weakly | 97.737593 | 0.10882 | 100.0 |
| 42 | SDI 42 | 0.774371 | 0.0105038 | 41.3727 | Non | Substrate | 97.469648 | 0.646074 | 100.0 |
| 43 | SDI 43 | 1.00737 | 0.00257049 | 43.4164 | Non | Substrate | 97.259474 | 1.00932 | 97.182894 |
| 44 | SDI 44 | 0.878706 | 0.00239627 | 39.4464 | Non | Substrate | 97.259474 | 4.56759 | 99.857654 |
| 45 | SDI 45 | 0.498909 | 0.0720876 | 53.4665 | Non | Substrate | 98.914988 | 0.177566 | 93.721844 |
| 46 | SDI 46 | 1.17532 | 0.0253862 | 36.7408 | Non | Substrate | 97.923347 | 8.85258 | 96.202468 |
| 47 | SDI 47 | 0.196299 | 0.0529024 | 27.6327 | Non | Weakly | 99.192985 | 0.0495572 | 87.294652 |
| 48 | SDI 48 | 0.179174 | 0.0424105 | 46.652 | Non | Substrate | 99.894819 | 0.16508 | 78.587316 |
| 49 | SDI 49 | 0.309947 | 0.00694488 | 49.5261 | Inhibitor | Weakly | 98.865884 | 6.18758 | 33.546459 |
| 50 | SDI 50 | 0.822955 | 0.00295984 | 36.4504 | Non | Substrate | 98.387506 | 0.16892 | 100.0 |
| 51 | Selegiline | 3.80237 | 181076 | 33.8469 | Non | Non | 100 | 245.483 | 87.698776 |

**Molecular docking studies**

*In silico* docking studies were performed using AutoDock 4.2 to identify the inhibiting potential of 49 SDI against monoamine oxidase (MAO- A) (PDB ID: 2BXR) and indoleamine 2,3-dioxygenase (IDO1) (PDB ID: 6E35). In the docking studies, if a compound shows lesser binding energy compared to the standard it proves that the compound has higher activity. The docking poses were ranked according to their docking scores. Binding energy of the individual compounds were calculated using the following formula,

Binding energy = A+B+C-D

Where, A denotes the final intermolecular energy + vanderWaals energy (vdW) + hydrogen bonds + desolvation energy + electrostatic energy (kcal/mol). B denotes the final total internal energy (kcal/mol). C denotes the torsional free energy (kcal/mol) and D denotes the unbound system's energy (kcal/mol).

***In silico* docking with enzymes Monoamine oxidase and Indoleamine 2,3-dioxygenase**

All the 49 compounds showed binding energy ranging from -7.59 kcal/mol to -5.14 kcal/mol, and -7.62 to -5.43 kcal/mol against MAO- A and IDO1 respectively. The standard drug Selegiline showed binding energy -5.09 kcal/mol and -5.29 kcal/mol with MAO- A and IDO1 respectively.

The other two parameters like inhibitory constant and intermolecular energy were also evaluated. Both inhibitory constant and intermolecular energy was directly proportional to the binding energy and the compounds showed inhibitory constant ranging from 334.47 nM to 2.0 μM and 213.58 nM to 2.58 μM against MAO- A and IDO1 respectively and that of standard drug Selegiline showed 184.97 μM and 132.95 μM, respectively.

The SDI showed intermolecular energy ranging from -11.22 and -4.41 kcal/mol against MAO and -10.36 and -5.35 kcal/mol against IDO1. The standard drug Selegiline showed intermolecular energy -6.29 kcal/mol against MAO- A and -6.48 kcal/mol for IDO.

Based on the binding energy, inhibitory constant and intermolecular energy, two compounds SDI 3 and SDI 6 were selected and synthesized for *in vitro* studies. Here, SDI 6 was found to possess excellent (low) binding energy, inhibitory constant and intermolecular energy whereas SDI 3 was found to possess poor (high) binding energy, inhibitory constant and intermolecular energy when compare to rest of selected compound and the standard. The

binding energy, inhibitory constant and intermolecular energy of 50 SDI compounds against enzyme MAO- A and IDO1 were tabulated (Table 8, 9).

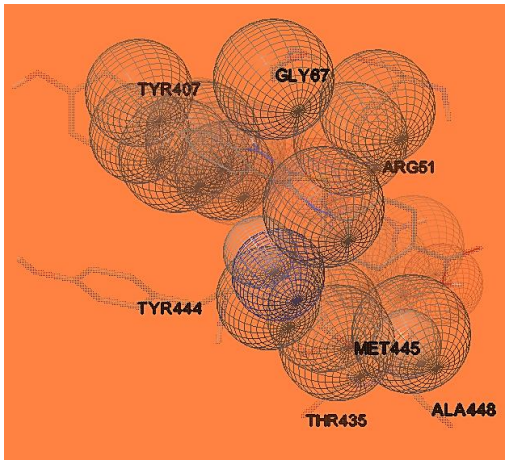
Based on the docking studies and compounds availability, two compounds (SDI 1 and SDI 6) with varying inhibition potential towards the enzyme targets were selected and synthesized for further *in vitro* studies. The compounds SDI 3 and SDI 6 showed good binding energy, inhibitory constant and intermolecular energy.

**Table 8: Docking results of SDI and standard against MAO- A (PDB ID– 2BXR)**

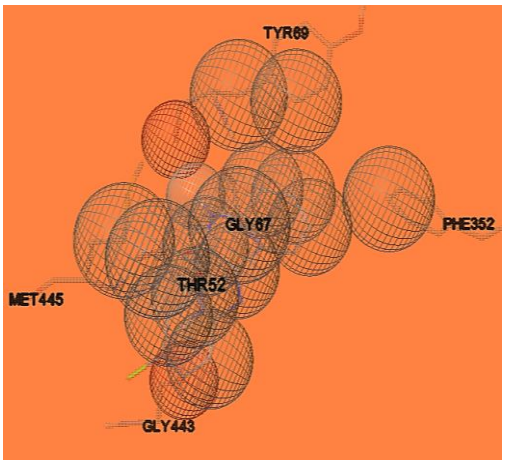
| Compound Code | Binding energy (kcal/mol) | Inhibitory constant (µM) (*- nM, ** - mM) | Intermolecular energy (kcal/mol) | Compound Code | Binding energy (kcal/mol) | Inhibitory constant (µM) (*- nM, ** - mM | Intermolecular energy (kcal/mol) |
|---|---|---|---|---|---|---|---|
| SDI 1 | -6.83 | 334.47* | -11.22 | SDI 26 | -7.57 | 2.8 | -8.47 |
| SDI 2 | -6.77 | 2.0 | -10.46 | SDI 27 | -7.27 | 4.66 | -8.77 |
| **SDI 3** | **-7.3** | **4.45** | **-9.99** | SDI 28 | -7.0 | 3.65 | -7.56 |
| SDI 4 | -6.55 | 2.93 | -9.93 | SDI 29 | -6.87 | 7.63 | -10.26 |
| SDI 5 | -7.46 | 3.4 | -10.14 | SDI 30 | -6.71 | 8.29 | -9.62 |
| **SDI 6** | **-7.59** | **2.93** | **-10.28** | SDI 31 | -7.43 | 3.59 | -8.32 |
| SDI 7 | -6.86 | 320.73* | -9.75 | SDI 32 | -6.6 | 4.58 | -7.65 |
| SDI 8 | -5.32 | 224.15 | -10.22 | SDI 33 | -7.39 | 3.85 | -8.88 |
| SDI 9 | -5.14 | 112.43 | -8.76 | SDI 34 | -7.32 | 7.32 | -9.51 |
| SDI 10 | -7.52 | 3.05 | -8.42 | SDI 35 | -7.31 | 4.4 | -8.5 |
| SDI 11 | -7.04 | 6.57 | -7.94 | SDI 36 | -6.75 | 11.34 | -8.24 |
| SDI 12 | -7.56 | 2.86 | -9.05 | SDI 37 | -7.09 | 6.35 | -9.18 |
| SDI 13 | -7.01 | 3.35 | -7.53 | SDI 38 | -7.18 | 5.46 | -9.86 |
| SDI 14 | -6.68 | 12.65 | -8.17 | SDI 39 | -7.55 | 2.9 | -9.34 |
| SDI 15 | -6.23 | 10.27 | -9.05 | SDI 40 | -7.18 | 5.42 | -8.97 |
| SDI 16 | -6.91 | 8.65 | -8.1 | SDI 41 | -7.01 | 6.35 | -7.36 |
| SDI 17 | -7.19 | 5.36 | -8.98 | SDI 42 | -6.86 | 7.25 | -8.25 |
| SDI 18 | -7.29 | 4.55 | -9.08 | SDI 43 | -7.11 | 6.35 | -6.38 |
| SDI 19 | -6.62 | 14.12 | -9.0 | SDI 44 | -6.78 | 8.36 | -9.05 |
| SDI 20 | -6.51 | 16.89 | -8.3 | SDI 45 | -6.32 | 7.69 | -9.84 |
| SDI 21 | -7.32 | 4.29 | -9.11 | SDI 46 | -6.80 | 8.34 | -10.61 |
| SDI 22 | -7.13 | 5.96 | -8.32 | SDI 47 | -6.46 | 6.57 | -4.41 |
| SDI 23 | -7.48 | 3.31 | -8.37 | SDI 48 | -7.23 | 8.25 | -9.27 |
| SDI 24 | -7.23 | 5.04 | -8.72 | SDI 49 | -7.27 | 9.63 | -8.36 |
| SDI 25 | -7.54 | 2.95 | -8.74 | SDI 50 | -6.95 | 8.57 | -7.65 |
| Selegiline | -5.09 | 184.97 | -6.29 | | | | |

**Table 9: Docking results of SDI and standard against IDO1 (PDB ID – 6E35)**

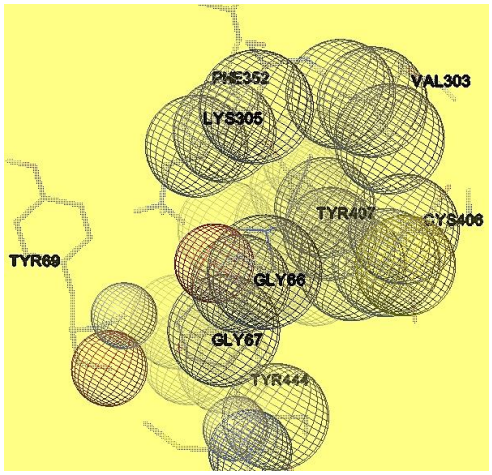
| Compound Code | Binding energy (kcal/mol) | Inhibitory constant (μM) | Intermolecular energy (kcal/mol) | Compound Code | Binding energy (kcal/mol) | Inhibitory constant (μM) (*- nM, ** - mM | Intermolecular energy (kcal/mol) |
|---|---|---|---|---|---|---|---|
| SDI 1 | -6.9 | 8.82 | -5.35 | SDI 26 | -7.01 | 7.12 | -7.61 |
| SDI 2 | -6.71 | 12.08 | -7.31 | SDI 27 | -7.54 | 2.97 | -8.43 |
| **SDI 3** | **-7.02** | **7.19** | **-7.91** | SDI 28 | -7.13 | 4.36 | -7.53 |
| SDI 4 | -6.8 | 10.32 | -7.1 | SDI 29 | -6.98 | 5.10 | -7.10 |
| SDI 5 | -7.5 | 3.18 | -8.1 | SDI 30 | -7.24 | 8.68 | -10.36 |
| **SDI 6** | **-7.62** | **2.58** | **-7.92** | SDI 31 | -6.85 | 9.53 | -7.74 |
| SDI 7 | -7.55 | 2.95 | -8.44 | SDI 32 | -7.28 | 4.6 | -8.18 |
| SDI 8 | -7.51 | 3.14 | -8.4 | SDI 33 | -7.12 | 6.0 | -8.62 |
| SDI 9 | -6.81 | 10.23 | -7.7 | SDI 34 | -7.3 | 4.49 | -8.49 |
| SDI 10 | -6.86 | 50.88 | -6.75 | SDI 35 | -7.31 | 4.39 | -8.5 |
| SDI 11 | -6.89 | 8.85 | -7.79 | SDI 36 | -6.86 | 9.43 | -8.35 |
| SDI 12 | -6.57 | 15.16 | -8.07 | SDI 37 | -5.85 | 51.34 | -7.94 |
| SDI 13 | -6.52 | 16.55 | -8.01 | SDI 38 | -5.43 | 10.64 | -8.11 |
| SDI 14 | -6.01 | 213.58 | -6.5 | SDI 39 | -7.32 | 4.34 | -9.11 |
| SDI 15 | -6.92 | 45.92 | -7.41 | SDI 40 | -6.88 | 9.03 | -8.67 |
| SDI 16 | -7.53 | 3.05 | -8.72 | SDI 41 | -6.89 | 8.32 | -7.56 |
| SDI 17 | -6.82 | 10.08 | -8.61 | SDI 42 | -7.23 | 8.25 | -9.27 |
| SDI 18 | -6.47 | 18.13 | -8.26 | SDI 43 | -7.27 | 9.63 | -8.36 |
| SDI 19 | -6.4 | 20.39 | -8.79 | SDI 44 | -6.95 | 8.57 | -7.65 |
| SDI 20 | -6.98 | 7.64 | -8.77 | SDI 45 | -7.23 | 8.25 | -9.27 |
| SDI 21 | -7.34 | 4.14 | -9.13 | SDI 46 | -6.86 | 7.25 | -8.25 |
| SDI 22 | -6.85 | 9.58 | -8.04 | SDI 47 | -7.11 | 6.35 | -6.38 |
| SDI 23 | -7.57 | 2.85 | -8.76 | SDI 48 | -6.78 | 8.36 | -9.05 |
| SDI 24 | -7.54 | 2.96 | -8.44 | SDI 49 | -6.32 | 7.69 | -9.84 |
| SDI 25 | -7.39 | 3.81 | -8.88 | SDI 50 | -7.02 | 8.25 | -7.50 |
| Selegiline | -5.29 | 132.95 | -6.48 | | | | |



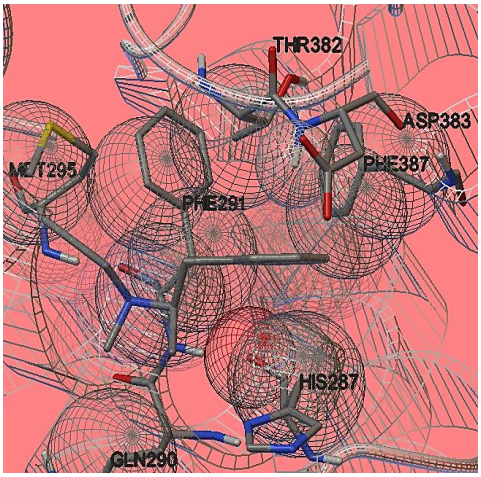

MAO against SDI 3



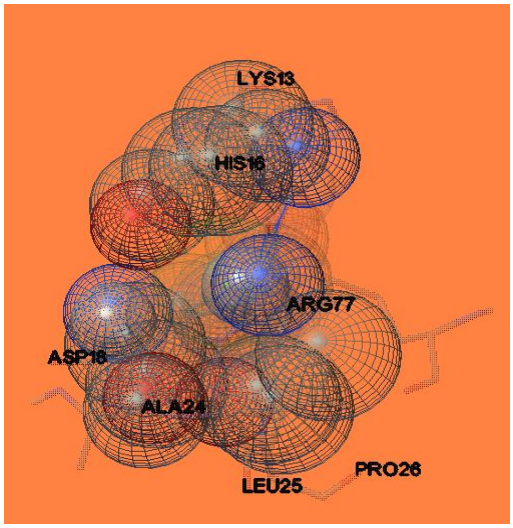

MAO against SDI 6



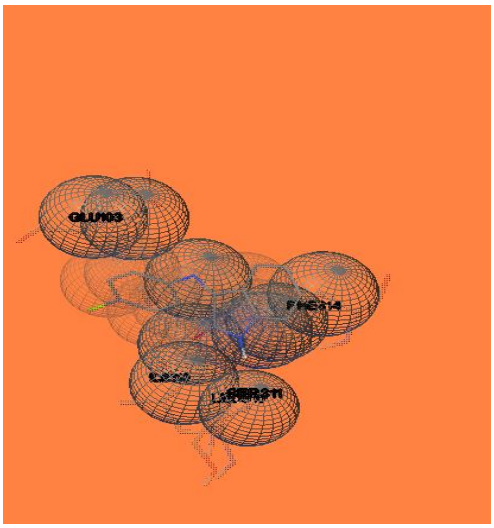

MAO against Selegiline




IDO against Selegiline




IDO against SDI 6



IDO against SDI 3

**Fig 9 : Binding images of selected compounds and standard against monoamine oxidase and indoleamine 2,3-dioxygenase**

The amino acid residues of the standard Selegiline and the selected test compounds were also found to be similar (Table - 10 & 11) and the docking images are shown in Fig. 9.

**Table10: Docking orientations of selected SDI and standard against MAO - A**

| Compound | Binding interaction with amino acid residue against monoamine oxidase |
|---|---|
| SDI 3 | **TYR 407**, **GLY 67**, ARG 51, **TYR 444**, ALA 448, THR 435, MET 445 |
| SDI 6 | GLY 443, MET 445, THR 52, **GLY 67**, **TYR 69**, **PHE 352** |
| Selegiline | **PHE 352**, LYS 305, VAL 303, **TYR 407**, CYS 406, GLY 66, **GLY 67**, **TYR 444**, **TYR 69** |

**Table 11: Docking orientations of selected SDI and standard against IDO1**

| Compound | Binding interaction with amino acid residue against indoleamine 2,3-dioxygenase |
|---|---|
| SDI 3 | **SER 167**, ALA 264, PHE 270, ARG 343, **HIS 346**, LEU 342, PHE 214, **VAL 170** |
| SDI 6 | TYR 126, **PHE 163**, **SER 167**, **VAL 170**, PHE 226, **ILE 217**, PHE 214, **HIS 346**, TYR 353, ILE 349, VAL 350 |
| Selegiline | **PHE163**, **SER 167**, VAL 166, **VAL 170**, **ILE 217**, PHE 214, **HIS 346**, ILE 346 |

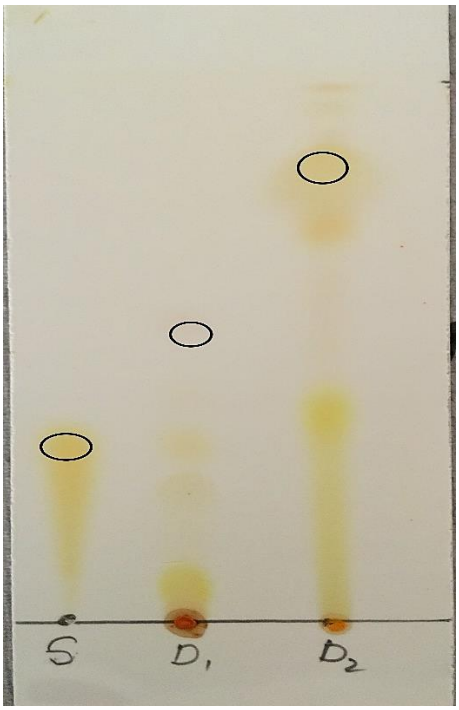
**PHYSICAL DATA OF THE SYNTHETIC DERIVATIVES OF ISATIN**

The percentage yield, melting point and $R_f$ values of pure isatin and the synthesized SDI compounds were provided in the table 12. The percentage yield of the synthesized compounds SDI 3 and SDI 6 were found to be 75.3% w/v and 72.4% w/v respectively. The melting point was determined by using MR-VIS visual melting range apparatus and it was found to be 200°C for isatin. The $R_f$ value was determined in TLC using solvent ethyl acetate: n-hexane (1:2) and was found to be 0.33 for isatin.

The melting point for SDI 3 was found to be 186°C and for SDI 6, was 198°C respectively. The $R_f$ values of synthesized compounds SDI 3 and 6 was 0.53 and 0.86 respectively. All the compounds were soluble in alcohol, benzene, acetone and insoluble in nonpolar organic solvents.

**Table 12: Physical data of Isatin and synthetic derivative of isatin**

| Compound | Molecular formula | Molecular weight (g/mol) | Percentage yield (% w/v) | Melting point (°C) | Rf value | Solubility |
|---|---|---|---|---|---|---|
| **Isatin** | $C_8H_5NO_2$ | 147.13 | - | 200 | 0.33 | **Soluble** – water, hot ethanol, benzene, acetone,ether-soluble **Insoluble** – nonpolar organic solvents |
| **SDI 3** | $C_{15}H_{10}N_2O_3$ | 266.25 | 75.3 | 186 | 0.53 | |
| **SDI 6** | $C_{14}H_9ClN_2O$ | 256.68 | 72.4 | 198 | 0.86 | |




S- Isatin

$D_1$- SDI 3

$D_2$- SDI 6

**Fig 10: TLC of Isatin, SDI 3 and SDI 6**

## SPECTRAL ANALYSIS OF COMPOUNDS

The compounds synthesized in the present investigation were established on the basis of the chemical data, UV and IR spectra. (Table 13,14. Fig. 10-14). The purity of all spectrum was established on single spot by TLC method.

### COMPOUND: SDI 3

**IUPAC name: 4-[(Z)-(2-oxo-1,2-dihydro-3H-indol-3-ylidene) amino] benzoic acid**

**UV spectroscopy**

$\lambda_{max}$ at 322 nm (Solvent used: Acetone)

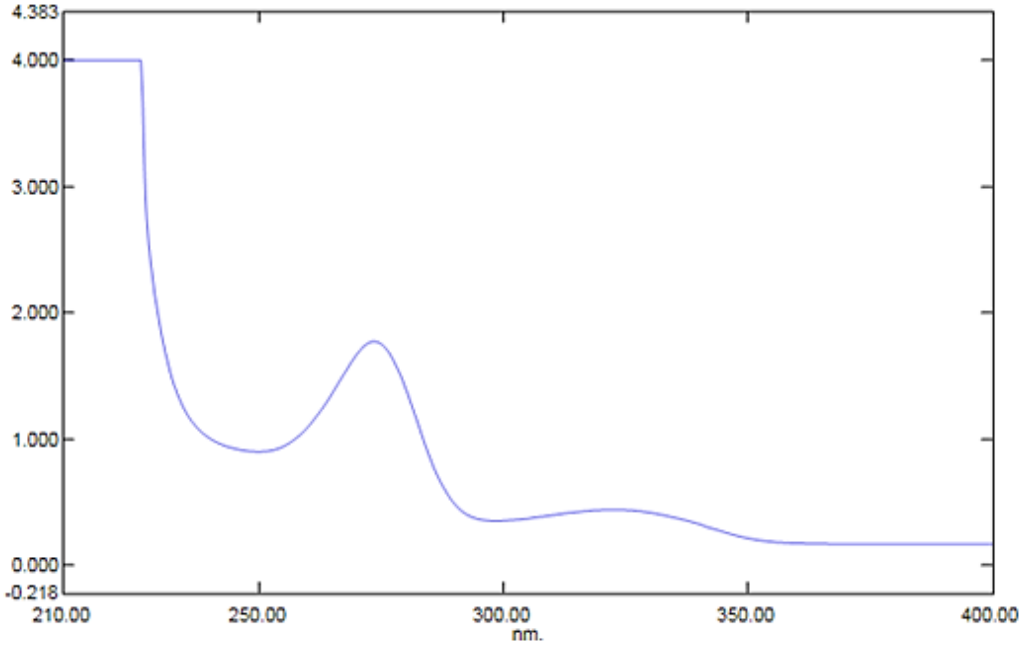



**Fig 11: UV absorption spectra of SDI 3**

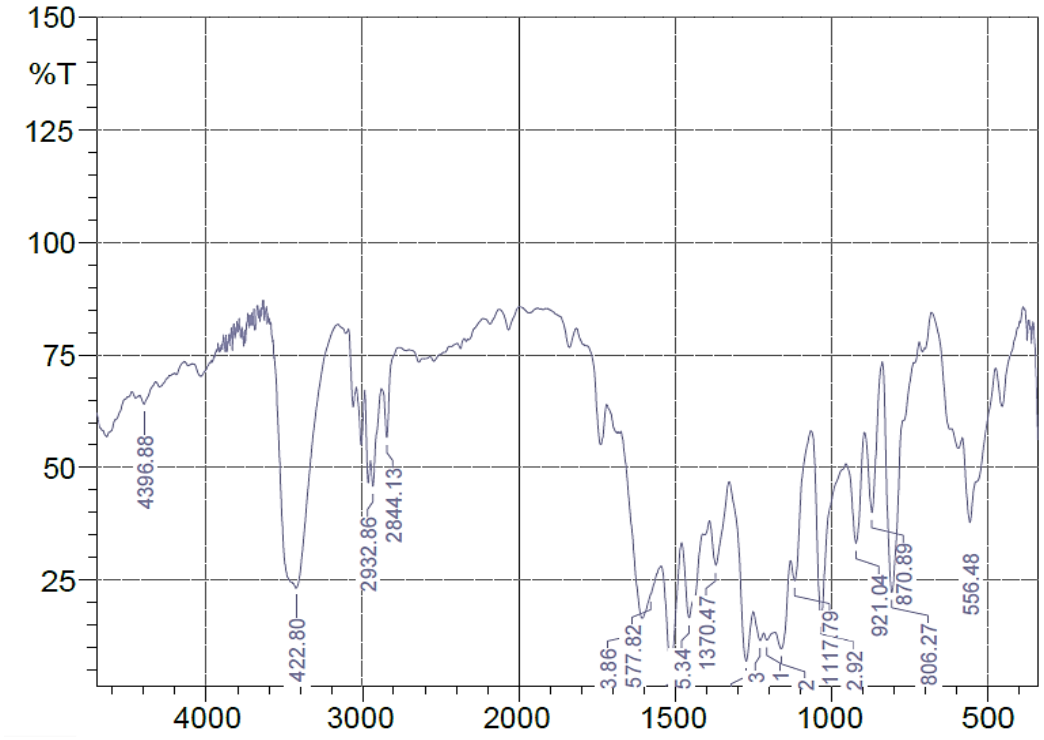



**Fig 12: IR spectrum of SDI 3**

**Table 13: Infra-red spectral data of SDI 3**

| S. No | Standard Stretching | Obtained Frequency | Types of stretching |
|---|---|---|---|
| 1 | 800-850 | 800 | C-H Stretching |
| 2 | 1200-1275 | 1273 | C=O Stretching |
| 3 | 1310-1390 | 1370 | O-H Stretching |
| 4 | 1500-1550 | 1513 | N-O Stretching |
| 5 | 3000-3400 | 3422 | N-H Stretching |

**COMPOUND: SDI 6**

**IUPAC name: (3Z)-3-[(4-chlorophenyl) imino]-1,3-dihydro-2H-indol-2-one.**

**UV spectroscopy**

$\lambda_{max}$ at 377 nm (Solvent used: Acetone)

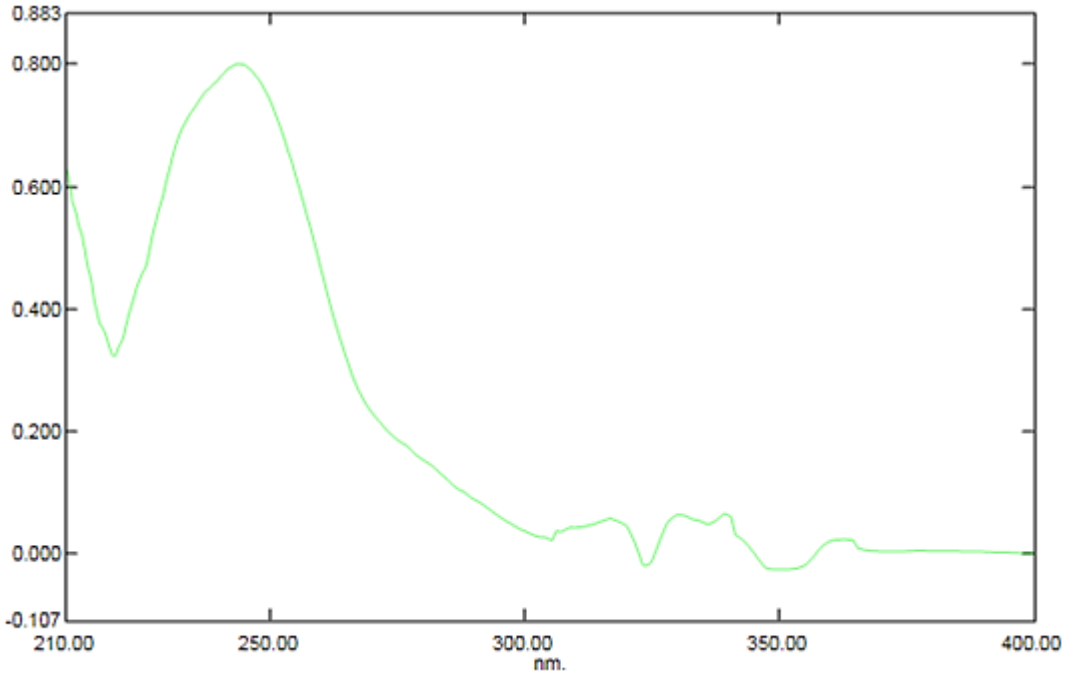



**Fig 13: UV absorption spectra of SDI 6**

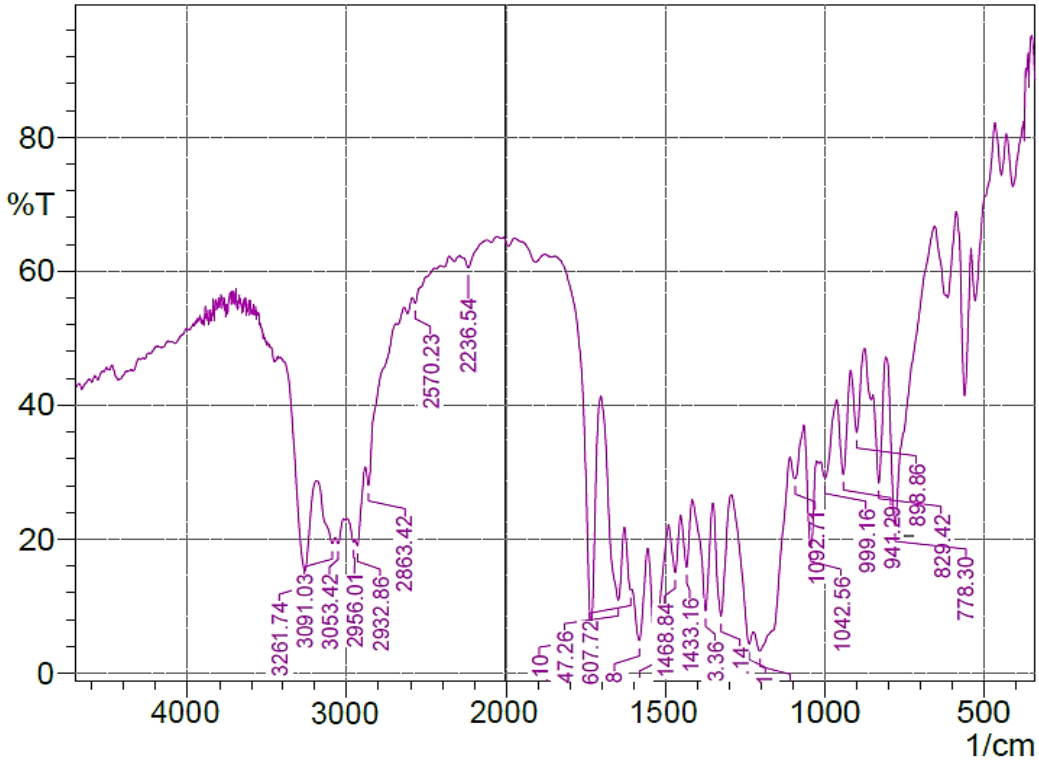



**Fig 14: IR spectrum of SDI 6**

**Table 14: Infra-red spectral data of SDI 6**

| S. No | Standard stretching | Obtained Stretching | Types of Stretching |
|---|---|---|---|
| 1 | 1020-1250 | 1202 | C-Cl Stretching |
| 2 | 1500-1550 | 1531 | N-O Stretching |
| 3 | 1715-1730 | 1733 | C=O Stretching |
| 4 | 2695-2865 | 2863 | C-H Stretching |
| 5 | 3000-3073 | 3091 | C-H Stretching |

## *IN VITRO* STUDY

### Evaluation of *in vitro* MAO- A inhibitory activity using SDI and standard Selegeline

Monoamine oxidases (MAO) are membrane-associated enzymes located specifically to the outer mitochondrial membrane. They are the major enzymes participating in the catabolism of monoamine neurotransmitters and related exogenous amines. Two isoforms of MAO exist, MAO-A and MAO-B, which differ in their substrate specificity, inhibitor selectivity and tissue distribution. Selective MAO-A inhibitors are useful in the therapy of depression and anxiety whereas MAO-B inhibitors are often used in the treatment of Parkinson's and Alzheimer's diseases.

**Table 15: MAO-A inhibitory activity of different concentration of SDI 3, SDI 6 and Selegiline**

| Concentration (µg/ml) | PERCENTAGE INHIBITION (MEAN ± SEM) | | |
|---|---|---|---|
| | SDI 3 | SDI 6 | SELEGILINE |
| 100 | 10.50±0.22 | 19.14±0.17 | 23.00±0.13 |
| 200 | 16.32±0.23 | 25.66±0.26 | 35.16±0.15 |
| 400 | 39.01±0.11 | 42.89±0.11 | 51.33±0.21 |
| 800 | 51.48±0.10 | 55.45±0.25 | 71.38±0.28 |
| 1600 | 64.68±0.14 | 71.40±0.25 | 84.04±0.28 |
| $IC_{50}$ | 726µg/ml | 599 µg/ml | 358 µg/ml |

The SDI 3 and SDI 6 showed significant MAO-A inhibitory activity at varying concentrations (100, 200, 400, 800, 1600 µg/ml). There was a dose dependent increase in the percentage inhibition for all the concentrations. The SDI 3 at concentration of 100 µg/ml showed a percentage inhibition of 10.5 and for 1600 µg/ml it was 64.6. The $IC_{50}$ value for SDI 3 was found to be 726 µg/ml. In the case of SDI 6, at a concentration of 100 µg/ml showed a percentage inhibition of 19.1 and for 1600 µg/ml it was 71.4.The $IC_{50}$ value was 599 µg/ml (Table 15).

Selegiline was used as the standard drug for the determination of MAO-A inhibitory activity. The concentration of selegiline varied from 100 to 1600 µg/ml. Selegiline at

concentration 100 µg/ml exhibited a percentage inhibition of 23.0 and for 1600 µg/ml it was found to be 84.04. A graded increase in percentage of inhibition was observed for the increase in the concentration of selegiline. The $IC_{50}$ value of selegiline was found to be 358 µg/ml. All determinations were done in triplicate and the mean values were determined.

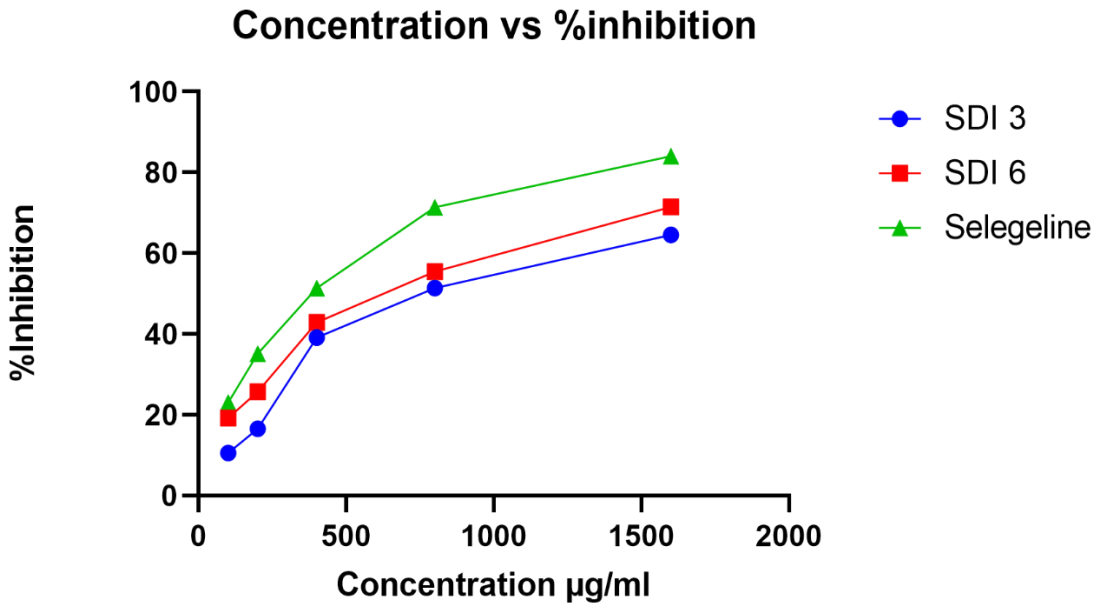



**Fig 15 : Percentage inhibition of MAO –A inhibitory activity of SDI derivatives and selegiline**

## ACUTE ORAL TOXICITY STUDIES

### Acute oral toxicity studies and selection of dose for *in vivo* studies

Acute oral toxicity was carried out as per OECD guidelines 420 employing fixed dose procedure for selecting the dose for biological activity. No toxic effect was observed in sighting study with a dose of 5 mg/kg, 50 mg/kg, 300 mg/kg and 2000 mg/kg of test compounds. In acute oral toxicity studies, the different parameters namely respiratory, motor, muscle tone, ocular sign, refluxes, skin, cardiovascular, piloerection, gastrointestinal sign were observed. There were no changes in normal behavioural pattern and no signs and symptoms of toxicity and mortality were observed. The live phase observations such as food intake, body weight, water consumption, home cage activity was also seen to be normal. It was found that the animals were safe up to a maximum dose of 2000 mg/kg of body weight. As per the OECD 420 guidelines, the synthetic derivatives of isatin SDI 3 and SDI 6 can be included in the category 5 or unclassified category of globally harmonized classification system (GHS). Hence, based on these results, the SDI 3 and SDI 6 were considered non-toxic and $1/10^{th}$ and $1/20^{th}$ dose were used for the biological evaluation (antidepressant activity) and the studies were conducted at dose levels of 100 and 200 mg/kg body weight for low and high dose respectively. (Table 16 - 23).

**Table 16: Observation for Acute Oral Toxicity study of test compound (SDI 3)**

| Parameters observed | | 0 h | 0.5h | 1 h | 2 h | 4 h | Day 2&3 | Day 4&5 | Day 6&7 | Day 8&9 | Day 10&11 | Day 12&13 | Day 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Respiratory** | Dyspnea | - | - | - | - | - | - | - | - | - | - | - | - |
| | Apnea | - | - | - | - | - | - | - | - | - | - | - | - |
| | Nostril discharges | - | - | - | - | - | - | - | - | - | - | - | - |
| **Motor activity** | Tremor | - | - | - | - | - | - | - | - | - | - | - | - |
| | Hyper activity | - | - | - | - | - | - | - | - | - | - | - | - |
| | Hypo activity | - | - | - | - | - | - | - | - | - | - | - | - |
| | Ataxia | - | - | - | - | - | - | - | - | - | - | - | - |
| | Jumping | - | - | - | - | - | - | - | - | - | - | - | - |
| | Catalepsy | - | - | - | - | - | - | - | - | - | - | - | - |
| | Locomotor activity | - | - | - | - | - | - | - | - | - | - | - | - |
| **Reflexes** | Corneal reflex | - | - | - | - | - | - | - | - | - | - | - | - |
| | Pinna reflex | - | - | - | - | - | - | - | - | - | - | - | - |
| | Righting reflex | - | - | - | - | - | - | - | - | - | - | - | - |
| **Convulsion** | Tonic and clonic convulsion | - | - | - | - | - | - | - | - | - | - | - | - |
| **Muscle tone** | Hypertonia | - | - | - | - | - | - | - | - | - | - | - | - |
| | Hypotonia | - | - | - | - | - | - | - | - | - | - | - | - |
| **Ocular sign** | Lacrimation | - | - | - | - | - | - | - | - | - | - | - | - |
| | Miosis | - | - | - | - | - | - | - | - | - | - | - | - |
| | Mydriasis | - | - | - | - | - | - | - | - | - | - | - | - |
| | Ptosis | - | - | - | - | - | - | - | - | - | - | - | - |

| Parameters observed | | 0 h | 0.5h | 1 h | 2 h | 4 h | Day 2&3 | Day 4&5 | Day 6&7 | Day 8&9 | Day 10&11 | Day 12&13 | Day 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Skin** | Edema | - | - | - | - | - | - | - | - | - | - | - | - |
| | Skin and fur | - | - | - | - | - | - | - | - | - | - | - | - |
| | Erythema | - | - | - | - | - | - | - | - | - | - | - | - |
| **Cardiovascular signs** | Bradycardia | - | - | - | - | - | - | - | - | - | - | - | - |
| | Tachycardia | - | - | - | - | - | - | - | - | - | - | - | - |
| **Piloerection** | Contraction of erectile tissue of hair | - | - | - | - | - | - | - | - | - | - | - | - |
| **Gastro intestinal signs** | Diarrhoea | - | - | - | - | - | - | - | - | - | - | - | - |

**Table 17: Live phase observation**

| LIVE PHASE ANIMALS | OBSERVATIONS |
|---|---|
| Body weight every day | Normal |
| Food consumption daily | Normal |
| Water consumption daily | Normal |
| Home cage activity | Normal |

**Table 18: Mortality record of test compound SDI 3 for Acute oral toxicity study**

| Dose | Sighting study | | | | Main study | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 5 mg/kg | 50 mg/kg | 300 mg/kg | 2000 mg/kg | 2000 mg/kg | 2000 mg/kg | 2000 mg/kg | 2000 mg/kg | 2000 mg/kg |
| **No. of animals** | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| **Body weight (g)** | 130 | 145 | 140 | 150 | 145 | 150 | 140 | 140 | 150 |
| **Sex** | Female | Female | Female | Female | Female | Female | Female | Female | Female |
| **30 min** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **1 h** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **2 h** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **3 h** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **4 h** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 1** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 2** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 3** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 4** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 5** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 6** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 7** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 8** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 9** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |

**Table 19: Mortality record of test compound SDI 3 for Acute oral toxicity study**

| Dose | Sighting study | | | | Main study | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 5 mg/kg | 50 mg/kg | 300 mg/kg | 2000 mg/kg | 2000 mg/kg | 2000 mg/kg | 2000 mg/kg | 2000 mg/kg | 2000 mg/kg |
| **No. of animals** | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| **Body weight (g)** | 130 | 145 | 140 | 150 | 145 | 150 | 140 | 140 | 150 |
| **Sex** | Female | Female | Female | Female | Female | Female | Female | Female | Female |
| **Day 10** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 11** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 12** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 13** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 14** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Mortality** | 0/1 | 0/1 | 0/1 | 0/1 | 0/5 | | | | |

**Note:** Acute toxicity study of compound was performed and it was non-toxic up to 2000 mg/kg dose.

**Table 20: Observation for Acute Oral Toxicity study of test compound (SDI 6)**

| Parameters observed | | 0 h | 0.5h | 1 h | 2 h | 4 h | Day 2&3 | Day 4&5 | Day 6&7 | Day 8&9 | Day 10&11 | Day 12&13 | Day 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Respiratory** | Dyspnea | - | - | - | - | - | - | - | - | - | - | - | - |
| | Apnea | - | - | - | - | - | - | - | - | - | - | - | - |
| | Nostril discharges | - | - | - | - | - | - | - | - | - | - | - | - |
| **Motor activity** | Tremor | - | - | - | - | - | - | - | - | - | - | - | - |
| | Hyper activity | - | - | - | - | - | - | - | - | - | - | - | - |
| | Hypo activity | - | - | - | - | - | - | - | - | - | - | - | - |
| | Ataxia | - | - | - | - | - | - | - | - | - | - | - | - |
| | Jumping | - | - | - | - | - | - | - | - | - | - | - | - |
| | Catalepsy | - | - | - | - | - | - | - | - | - | - | - | - |
| | Locomotor activity | - | - | - | - | - | - | - | - | - | - | - | - |
| **Reflexes** | Corneal reflex | - | - | - | - | - | - | - | - | - | - | - | - |
| | Pinna reflex | - | - | - | - | - | - | - | - | - | - | - | - |
| | Righting reflex | - | - | - | - | - | - | - | - | - | - | - | - |
| **Convulsion** | Tonic and clonic convulsion | - | - | - | - | - | - | - | - | - | - | - | - |
| **Muscle tone** | Hypertonia | - | - | - | - | - | - | - | - | - | - | - | - |
| | Hypotonia | - | - | - | - | - | - | - | - | - | - | - | - |
| **Ocular sign** | Lacrimation | - | - | - | - | - | - | - | - | - | - | - | - |
| | Miosis | - | - | - | - | - | - | - | - | - | - | - | - |
| | Mydriasis | - | - | - | - | - | - | - | - | - | - | - | - |
| | Ptosis | - | - | - | - | - | - | - | - | - | - | - | - |

| Parameters observed | | 0 h | 0.5h | 1 h | 2 h | 4 h | Day 2&3 | Day 4&5 | Day 6&7 | Day 8&9 | Day 10&11 | Day 12&13 | Day 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Skin** | Edema | - | - | - | - | - | - | - | - | - | - | - | - |
| | Skin and fur | - | - | - | - | - | - | - | - | - | - | - | - |
| | Erythema | - | - | - | - | - | - | - | - | - | - | - | - |
| **Cardiovascular signs** | Bradycardia | - | - | - | - | - | - | - | - | - | - | - | - |
| | Tachycardia | - | - | - | - | - | - | - | - | - | - | - | - |
| **Piloerection** | Contraction of erectile tissue of hair | - | - | - | - | - | - | - | - | - | - | - | - |
| **Gastro intestinal signs** | Diarrhoea | - | - | - | - | - | - | - | - | - | - | - | - |

**Table 21: Live phase observation**

| LIVE PHASE ANIMALS | OBSERVATIONS |
|---|---|
| 1. Body weight every day | Normal |
| 2. Food consumption daily | Normal |
| 3. Water consumption daily | Normal |
| 4. Home cage activity | Normal |

**Table 22: Mortality record of test compound SDI 6 for Acute oral toxicity study**

| Dose | Sighting study | | | | Main study | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 5 mg/kg | 50 mg/kg | 300 mg/kg | 2000 mg/kg | 2000 mg/kg | 2000 mg/kg | 2000 mg/kg | 2000 mg/kg | 2000 mg/kg |
| **No. of animals** | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| **Body weight (g)** | 140 | 145 | 140 | 150 | 145 | 150 | 140 | 155 | 150 |
| **Sex** | Female | Female | Female | Female | Female | Female | Female | Female | Female |
| **30 min** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **1 h** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **2 h** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **3 h** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **4 h** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 1** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 2** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 3** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 4** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 5** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 6** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 7** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 8** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 9** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |

**Table 23: Mortality record of test compound SDI 6 for Acute oral toxicity study**

| Dose | Sighting study | | | | Main study | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 5 mg/kg | 50 mg/kg | 300 mg/kg | 2000 mg/kg | 2000 mg/kg | 2000 mg/kg | 2000 mg/kg | 2000 mg/kg | 2000 mg/kg |
| **No. of animals** | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| **Body weight (g)** | 140 | 145 | 140 | 150 | 145 | 150 | 140 | 155 | 150 |
| **Sex** | Female | Female | Female | Female | Female | Female | Female | Female | Female |
| **Day 10** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 11** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 12** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 13** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Day 14** | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil | Nil |
| **Mortality** | 0/1 | 0/1 | 0/1 | 0/1 | 0/5 | | | | |

**Note:** Acute toxicity study of compound was performed and it was non-toxic up to 2000 mg/kg dose.

## BEHAVIOURAL ASSESSMENT

### Treatment of rats exposed to CUMS with SDI 3 and SDI 6 in despair swim test

The immobility time of rats during despair swim test were evaluated (Table 24). The results showed that in week 0, the time at which animals were immobile in water ranged from 92.33±4.66 to 106.33±2.74 sec with no significant difference. From week 1, animals in group II to VII were exposed to CUMS and immobility time prolonged with considerable difference. ($P > 0.05$). From week 4 onwards, the treatment groups showed significant difference with decrease in immobility time of treatment groups (ranging from 143.0±6.49 to 167.66±3.98 sec) when compared to negative control group (186.5±4.42 sec). At week 6, SDI 6 at low dose exhibited immobility time of 126.83±4.77 sec and high dose showed immobility time of 110.33±2.61sec and values were found to be statistically significant ($P < 0.01$). SDI 3 and standard drug showed immobility time at 139.83±4.77 sec and 107.83±5.95 sec respectively ($P < 0.01$).

### Measurement of immobility time in tail suspension test for SDI 3 and SDI 6 using CUMS model.

During week 0, all group of animals showed decrease in immobility time in tail suspension test. The immobility time of rats in tail suspension test ranged from 75.33±3.34 sec to 92.16±3.4 sec. From week 1 to 3, the immobility time gradually increased in group II to VII animals. The negative control showed significant increase in immobilitytime in week 3 (143.83±6.1 sec) when compared to control group (94.83±4.47 sec). In treatment groups, immobility time increased up to the end of 3$^{rd}$ week and the values were found to be non-significant ($P > 0.05$) when compared to negative control group. After 4 weeks of treatment with SDI 3, SDI 6 and standard, the immobility time decreased with significant difference ($P <0.01$). By the end of week 6, negative control group had shown immobility time of 188.16±7.13 sec increase in comparison to control group (92.66±10.57 sec) and was found to be significant. SDI 3 and 6 showed a dose dependent decrease ($P <0.01$) in immobility time when compared to negative control group. Standard drug also showed a significant decrease in immobility time in rats by the end of week 6 (88.33±4.08 sec). SDI 6 low dose (95.43±6.45 sec) and high dose (90.83±5.03 sec) produced more significant results in immobility time when compared to SDI 3 low and high doses (98.66±8.16 sec and 91.33±3.26 sec) respectively. (Table 25)

**Treatment of rats exposed to CUMS with SDI 3 and SDI 6 in elevated plus maze**

Week 0 observation showed that the animals in group I to VII spent more time in open arm (ranging from 250.33±12.97 to 277±3.81 sec) and less time in closed arm (ranging from 23±3.82 to 49.66±13.02 sec) with non-significant difference. In week 1-3, the time spent by animals in closed arm has increased and time spent in open arm has decreased in group II to VII and the level of significance was found to be ($P > 0.05$). At week 4 and 5, the treatment groups had shown the significant difference in time spent by animals in closed and open arm when compared to negative control group ($P < 0.01$). At week 6, treatment with SDI 3 and 6 at low and high dose spent more time in open arm (228.83±4.81 and 248.33±3.42; 230.5±3.67 and 264±5.63) and results were found to be significant in dose dependent manner ($P < 0.01$) but SDI 3 markedly shows the best reversal of time spent in open arm when compared to SDI 6. Standard group also showed the significant difference in time spent by animals in open (260.33±3.23) and closed arm (39.66±3.24). Similarly, negative control group showed significant difference in time spent in open and closed arms ($P < 0.01$) (Table 26, 27).

**Treatment of rats exposed to CUMS with SDI 3 and SDI 6 in sucrose preference test**

The percentage of sucrose preference in rats of groups I to VII were evaluated for week 0 to week 6 (Table 28). The results showed that in week 0 all the rats prefer 75.39±3.15 to 84.37±2.54% sucrose than water with no significant difference. From week 1 to week 6, rats were exposed to CUMS except control group, and showed decrease in the preference to sucrose solution by animals in all groups except control and no significant difference ($P > 0.05$) was noticed. At week 4, the preference of sucrose is slightly increased in treatment groups when compared to week 3 and for negative control group the preference of sucrose in decreased (48.34±4.65%). At week 5 and 6, the preference to sucrose was further increased in treatment groups in dose dependent manner. Percentage sucrose preference was found to be statistically significant ($P < 0.01$) when compared to control. At week 6, the increase in sucrose preference in the treatment group animals ($P < 0.01$) were seen whereas negative control shows significant decrease in sucrose preference (21.48±4.56%) when compared to control group (78.32±3%). The compounds SDI 3 and 6 showed dose dependent increase with significant difference ($P < 0.01$). SDI 6 showed more preference to sucrose in animals than SDI 3. Standard also showed gradual increase in sucrose preference in week 6 when compared to that of the treatment group.

**Effect of SDI 3 and 6 in social interaction in rats exposed to CUMS**

Animals in group I-VII were observed for social interaction from week 0 to week 6 (Table 29). In week 0, social interaction test revealed a range of 80.14±4.23 to 87.09±2.34 sec and the values were significant. In week 1 to 3 after CUMS, the social interaction time in animals increased significantly ($P < 0.05$) except control group. During week 4, the interaction time gradually increased in group III to VII ranging from 80.17±3.81 to 84.67±1.23 sec with ($P < 0.05$). At week 6, the negative control group showed significant decrease in interaction time (65.86±2.19 sec) when compared to control group (94.04±1.27 sec) and also SDI 3 and 6 showed increase in social interaction time at $P < 0.01$ when compared to negative control group. Among two test drugs SDI 6 had shown increased interaction time in rats than SDI 3. The standard drug showed increased interaction time (90.18±3.32 sec) when compared to the test drugs.

**Table 24: Immobility time in despair swim test in rats exposed to CUMS**

| Group | Immobility time (sec) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Week 0** | **Week 1** | **Week 2** | **Week 3** | **Week 4** | **Week 5** | **Week 6** |
| Control | 102.16±3.84 | 103.16±3.58 | 104.16±3.21 | 103.83±3.78 | 103.5±1.94 | 104.33±2.78 | 105.16±3.8 |
| Negative control | 106.33±2.74$^{ns}$ | 128.5±2.36$^{\#\#}$ | 152.16±2.21$^{\#\#}$ | 177.5±4.48$^{\#\#}$ | 186.5±4.42$^{\#\#}$ | 201.66±3.21$^{\#\#}$ | 220.33±2.18$^{\#\#}$ |
| SDI 3 – LD | 101.33±5.48$^{ns}$ | 122.5±4.87$^{ns}$ | 149.66±4.19$^{ns}$ | 176.33±3.49$^{ns}$ | 167.66±3.98$^{ns}$ | 156.66±3.72$^{**}$ | 139.83±2.62$^{**}$ |
| SDI 3 – HD | 98.33±4.72$^{ns}$ | 123.16±4.32$^{ns}$ | 152.83±4.97$^{ns}$ | 174.66±6.28$^{ns}$ | 158.16±7.61$^{**}$ | 135.33±6.52$^{**}$ | 117.5±5.88$^{**}$ |
| SDI 6 – LD | 102±2.39$^{ns}$ | 122.83±3.83$^{ns}$ | 149±4.32$^{ns}$ | 168.66±5.25$^{ns}$ | 156.66±5.73$^{**}$ | 142.5±5.09$^{**}$ | 126.83±4.77$^{**}$ |
| SDI 6– HD | 93.33±4.06$^{ns}$ | 115±3.39$^{ns}$ | 137.83±4.51$^{ns}$ | 164.33±4.95$^{ns}$ | 152.66±4.71$^{**}$ | 133.33±3.73$^{**}$ | 110.33±2.61$^{**}$ |
| Selegiline | 92.33±4.66$^{ns}$ | 119±5.02$^{ns}$ | 138.83±3.8$^{ns}$ | 160.33±6.24$^{ns}$ | 143±6.49$^{**}$ | 125.83±6.49$^{**}$ | 107.83±5.95$^{**}$ |

Values were expressed in mean ± SEM.

Data were analysed by one-way ANOVA followed by Dunnett's test. In negative control group, $^{\#\#}P < 0.01$ and $^{ns}P > 0.05$ as compared to control group. In treatment groups, $^{**}P < 0.01$ and $^{ns}P > 0.05$ compared to negative control group.

**Table 25: Immobility time in tail suspension test in rats exposed to CUMS**

| Group | Immobility time (sec) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Week 0 | Week 1 | Week 2 | Week 3 | Week 4 | Week 5 | Week 6 |
| Control | 88.5±3.39 | 95.83±5.57 | 97.5±5.45 | 94.83±5.77 | 95.5±4.47 | 96.33±4.49 | 92.66±10.57 |
| Negative control | 87.66±3.33$^{ns}$ | 102.66±4.63$^{ns}$ | 114.83±4.72$^{\#}$ | 132.33±6.59$^{\#\#}$ | 143.83±6.1$^{\#\#}$ | 168.12±3.99$^{\#\#}$ | 188.16±7.13$^{\#\#}$ |
| SDI 3 – LD | 83.83±3.17$^{ns}$ | 97.36±4.33$^{ns}$ | 110.16±4.67$^{ns}$ | 124.33±4.86$^{**}$ | 118.36±3.91$^{**}$ | 109.57±3.54$^{**}$ | 98.66±8.16$^{**}$ |
| SDI 3 – HD | 92.16±3.4$^{ns}$ | 103.33±4.14$^{ns}$ | 119.16±3.49$^{ns}$ | 133.33±3.55$^{ns}$ | 124.33±3.49$^{**}$ | 103.62±2.06$^{**}$ | 91.33±3.26$^{**}$ |
| SDI 6 – LD | 88.83±3.6$^{ns}$ | 100.12±3.66$^{ns}$ | 112.83±3.64$^{ns}$ | 126.66±3.22$^{ns}$ | 117.33±3.14$^{**}$ | 105.83±3.97$^{**}$ | 95.43±6.45$^{**}$ |
| SDI 6– HD | 83.5±2.5$^{ns}$ | 97.16±2.81$^{ns}$ | 120.16±4.24$^{ns}$ | 135.63±2.64$^{ns}$ | 123.33±2.33$^{**}$ | 106.5±2.76$^{**}$ | 90.83±5.03$^{**}$ |
| Selegiline | 75.33±3.34$^{ns}$ | 93.83±1.99$^{ns}$ | 115.5±3.11$^{ns}$ | 124.45±1.69$^{ns}$ | 119.16±1.4$^{**}$ | 108.33±1.52$^{**}$ | 88.33±4.08$^{**}$ |

Values were expressed in mean ± SEM.

Data were analysed by one-way ANOVA followed by Dunnett's test. In negative control group, $^{\#\#}P < 0.01$ and $^{ns}P > 0.05$ compared to control group. In treatment groups, $^{**}P < 0.01$ and $^{ns}P > 0.05$ compared to negative control group.

**Table 26: Effect of SDI 3 and 6 on time spent in open arm in elevated plus maze in rats exposed to CUMS**

| Group | Time spent in open arm (sec) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Week 0** | **Week 1** | **Week 2** | **Week 3** | **Week 4** | **Week 5** | **Week 6** |
| Control | 260.33±7.38 | 269.66±4.06 | 275.83±3.35 | 271.83±4.62 | 275±2.46 | 272.16±3.32 | 255.5±6.38 |
| Negative control | 250.33±12.97$^{ns}$ | 232.66±4.52$^{\#\#}$ | 184.66±3.09$^{\#\#}$ | 158.83±3.43$^{\#\#}$ | 140.16±4.8$^{\#\#}$ | 119.83±2.86$^{\#\#}$ | 110.33±2.23$^{\#\#}$ |
| SDI 3 – LD | 277±3.81$^{*}$ | 238±6.17 $^{ns}$ | 192.16±2.33 $^{ns}$ | 152.16±3.47 $^{ns}$ | 193.5±4.81$^{**}$ | 210.83±4.32$^{**}$ | 228.83±4.81$^{**}$ |
| SDI 3 – HD | 271.66±5.45 $^{ns}$ | 250.66±4.15$^{*}$ | 197.83±8.49 $^{ns}$ | 162.16±3.43 $^{ns}$ | 204.16±3.07$^{**}$ | 241.33±9.31$^{**}$ | 248.33±3.42$^{**}$ |
| SDI 6 – LD | 267.5±4.13 $^{ns}$ | 250.5±4.4$^{*}$ | 186.5±6.78 $^{ns}$ | 168.66±3.35 $^{ns}$ | 179.16±3.55$^{**}$ | 189.33±6.54$^{**}$ | 230.5±3.67$^{**}$ |
| SDI 6– HD | 272±4.96 $^{ns}$ | 246.83±2.76 $^{ns}$ | 185±10.87 $^{ns}$ | 154.66±6.98 $^{ns}$ | 189.33±4.25$^{**}$ | 214±6.46$^{**}$ | 264±5.63$^{**}$ |
| Selegiline | 262.66±4.38 $^{ns}$ | 247.33±4.59 $^{ns}$ | 175.5±5.42 $^{ns}$ | 149.5±3.41 $^{ns}$ | 181.66±3.22 $^{**}$ | 230.66±3.18$^{**}$ | 260.33±3.23$^{**}$ |

Value are expressed in mean ± SEM.

Data were analysed by one-way ANOVA followed by Dunnett's test. In negative control group, $^{\#\#}P < 0.01$ and $^{ns}P > 0.05$ compared to control. In treatment groups, $^{**}P < 0.01$, $^{*}P < 0.05$ and $^{ns}P > 0.05$ as compared to negative control.

**Table 27: Effect of SDI 3 and 6 on time spent in closed arm in elevated plus maze in rats exposed to CUMS**

| Group | Time spent in closed arm (sec) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Week 0 | Week 1 | Week 2 | Week 3 | Week 4 | Week 5 | Week 6 |
| Control | 39.66±7.41 | 30.33±4.07 | 24.16±3.36 | 28.16±4.64 | 25±2.47 | 27.83±3.33 | 44.5±6.4 |
| Negative control | 49.66±13.02 $^{ns}$ | 67.33±4.53$^{\#\#}$ | 115.33±3.1$^{\#\#}$ | 141.16±3.45$^{\#\#}$ | 159.83±4.82$^{\#\#}$ | 180.16±2.87$^{\#\#}$ | 189.66±2.23$^{\#\#}$ |
| SDI 3 – LD | 23±3.82$^{*}$ | 62±6.19 $^{ns}$ | 107.83±2.33 $^{ns}$ | 147.83±3.49 $^{ns}$ | 136.83±4.83 $^{**}$ | 92.16±4.34$^{**}$ | 71.16±4.83$^{**}$ |
| SDI 3 – HD | 28.33±5.47 $^{ns}$ | 49.33±4.16$^{*}$ | 102.16±8.53 $^{ns}$ | 137.83±3.45 $^{ns}$ | 95.83±3.08 $^{**}$ | 58.66±9.35$^{**}$ | 51.66±3.43$^{**}$ |
| SDI 6 – LD | 32.5±4.15 $^{ns}$ | 49.5±4.41$^{*}$ | 113.5±6.8 $^{ns}$ | 131.33±3.26 $^{ns}$ | 120.83±3.56$^{**}$ | 110.66±6.56$^{**}$ | 69.5±3.69$^{**}$ |
| SDI 6– HD | 28±4.98 $^{ns}$ | 53.16±2.77 $^{ns}$ | 115±10.41 $^{ns}$ | 145.33±7.01 $^{ns}$ | 110.66±4.27$^{**}$ | 86±6.48$^{**}$ | 36±5.65$^{**}$ |
| Selegiline | 37.33±4.04 $^{ns}$ | 52.66±4.61 $^{ns}$ | 124.5±5.44 $^{ns}$ | 150.5±3.42 $^{ns}$ | 118.33±3.23 $^{**}$ | 69.33±3.19$^{**}$ | 39.66±3.24$^{**}$ |

Values were expressed in mean ± SEM.

Data were analysed by one-way ANOVA followed by Dunnett's test. In negative control group, $^{\#\#}P < 0.01$ and $^{ns}P > 0.05$ as compared to control group. In treatment groups, $^{**}P < 0.01$, $^{*}P < 0.05$ and $^{ns}P > 0.05$ compared to negative control group.

**Table 28: Effect of SDI 3 and 6 on percentage of sucrose preference in rats exposed to CUMS**

| Group | Sucrose Preference (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Week 0 | Week 1 | Week 2 | Week 3 | Week 4 | Week 5 | Week 6 |
| Control | 75.39±3.15 | 78.17±4.36 | 82.19±1.64 | 79.37±3.57 | 78.15±3.78 | 80.49±2.04 | 78.32±3 |
| Negative control | 80.1±3.98$^{ns}$ | 70.29±2.58 $^{ns}$ | 51.65±1.73$^{\#\#}$ | 48.34±4.65$^{\#\#}$ | 41.29±3.46$^{\#\#}$ | 35.99±4.36$^{\#\#}$ | 21.48±4.56$^{\#\#}$ |
| SDI 3 – LD | 80.25±1.36 $^{ns}$ | 73.92±2.46 $^{ns}$ | 58.16±3.87 $^{ns}$ | 44.14±2.85 $^{ns}$ | 67.68±3.64$^{**}$ | 69.34±4.13$^{**}$ | 73.16±3.55$^{**}$ |
| SDI 3 – HD | 84.37±2.54 $^{ns}$ | 65.33±3.71 $^{ns}$ | 54.26±3.52 $^{**}$ | 41.48±2.74 $^{ns}$ | 62.84±2.53$^{**}$ | 77.36±2.53$^{**}$ | 83.56±4.57$^{**}$ |
| SDI 6 – LD | 81.2±2.01 $^{ns}$ | 73.65±1.98 $^{ns}$ | 55.9±3.52$^{*}$ | 47.16±2.41 $^{ns}$ | 65.12±3.47$^{**}$ | 74.22±1.06$^{**}$ | 78.11±3.55$^{**}$ |
| SDI 6– HD | 78.35±3.24 $^{ns}$ | 64.11±2.56 $^{ns}$ | 53.77±3.51$^{*}$ | 43.82±2.47 $^{ns}$ | 58.24±2.65$^{**}$ | 74.16±3.62$^{**}$ | 85.36±2.36$^{**}$ |
| Selegiline | 82.18±2.9 $^{ns}$ | 71.36±4.01 $^{ns}$ | 54.16±2.57$^{**}$ | 39.36±2.63 $^{ns}$ | 63.57±2.91$^{**}$ | 76.34±3.47$^{**}$ | 81.48±2.16$^{**}$ |

Values were expressed in mean ± SEM.

Data were analysed by one-way ANOVA followed by Dunnett's test. In negative control group, $^{\#\#}P < 0.01$ and $^{ns}P > 0.05$ compared to control. In treatment groups, $^{**}P < 0.01$, $^{*}P < 0.05$ and $^{ns}P > 0.05$ compared to negative control.

**Table 29: Effect of SDI 3 and 6 in social interaction time in rats exposed to CUMS**

| Group | Social Interaction time (sec) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Week 0** | **Week 1** | **Week 2** | **Week 3** | **Week 4** | **Week 5** | **Week 6** |
| Control | 83.21±3.27 | 85.03±1.21 | 86.14±1.31 | 88.18±1.67 | 92.19±2.34 | 95.21±2.97 | 94.04±1.27 |
| Negative control | 80.14±4.23$^{\#\#}$ | 82.07±2.31$^{\#\#}$ | 80.03±1.72$^{\#\#}$ | 79.26±2.78$^{\#\#}$ | 76.34±1.23$^{\#\#}$ | 70.67±4.53$^{\#\#}$ | 65.86±2.19$^{\#\#}$ |
| SDI 3 – LD | 86.34±4.23* | 85.12±3.21** | 83.23±1.09* | 80.34±4.16* | 82.06±3.21* | 84.34±2.03** | 87.19±4.09** |
| SDI 3 – HD | 82.61±2.91* | 80.34±3.94* | 78.04±1.21** | 75.21±1.76* | 80.17±3.81** | 83.04±8.13** | 86.04±3.08** |
| SDI 6 – LD | 87.09±2.34* | 86.23±1.07* | 82.54±2.21* | 79.43±2.21* | 81.04±2.45** | 84.11±1.59** | 87.34±1.50** |
| SDI 6– HD | 86.67±3.56* | 85.07±12.09* | 83.05±3.21* | 81.45±1.94* | 84.67±1.23** | 87.76±3.89** | 88.15±3.85** |
| Selegiline | 85.08±1.92* | 83.63±1.38* | 81.24±4.07* | 80.04±2.94* | 83.56±2.23* | 88.05±2.90* | 90.18±3.32* |

Values were expressed in mean ± SEM.

Data were analysed by one-way ANOVA followed by Dunnett's test. In negative control group, $^{\#\#}P < 0.01$, when compared to control.

In treatment groups, $^{*}P < 0.05$ and $^{**}P < 0.01$, when compared to negative control.

## BIOCHEMICAL ESTIMATION

### Serum parameters

### Effect of SDC 1 and 8 on serum corticosterone level in rat brain

Serum corticosterone level which is the biomarker for depression were estimated and the results are depicted in Table 30. The level of corticosterone in Group-II (11.54±0.32µg/ml) was found to increase significantly (P<0.01) when compared to level of corticosterone (3.12±0.07 µg/ml) in the Group-I. A significant (P<0.01) reduction of corticosterone level was found in the groups of rats treated with SDI 6 at doses of 100 and 200 mg/kg for 42 days (7.21±0.11 and 4.76±0.38 µg/ml respectively). SDI 3 showed a significant reduction in level of corticosterone with 7.93±0.43 µg/ml at low dose and 5.32±0.48 µg/ml at high dose significantly (P<0.01). The Selegiline treated group also offered a protective effect against stress-induced depression in rats by a significant (P<0.01) depletion of corticosterone level (4.32±0.29 µg/ml). From the results above it was clear that the SDI 6 possess antidepressant potential against stress-induced depression in rats.

**Table 30: Effect of SDI 3 and SDI 6 on serum corticosterone level in rat brain**

| Group | Serum corticosterone (µg/ml) |
|---|---|
| Control | 3.12±0.07 |
| Negative control | 11.54±0.32$^{\#\#}$ |
| SDI 3 – LD | 7.93±0.43$^{**}$ |
| SDI 3 – HD | 5.32±0.48$^{**}$ |
| SDI 6 – LD | 7.21±0.11$^{**}$ |
| SDI 6 – HD | 4.76±0.38$^{**}$ |
| Selegiline | 4.32±0.29$^{**}$ |

Values were expressed in mean ± SEM.

Data were analysed by one-way ANOVA followed by Dunnett's test. In negative control group, $^{\#\#}$P < 0.01 compared to control group. In treatment groups, **P<0.01, compared to negative control group.

**Tissue parameters**

**Effect of SDI 3 and SDI 6 on monoamine levels in the rat brain**

The standard graph of serotonin and norepinephrine in this study were constructed by plotting the concentration and absorbance of the two reference standards. The calibration curves were as follows: For serotonin, $y = 0.306x + 0.0052$ ($R^2 = 0.989$), for norepinephrine, $y = 1.261x + 0.0078$ ($R^2 = 0.9997$).

The level of monoamines in different groups were tabulated in Table 31. CUMS exposed rats showed significant ($P<0.01$) decrease in the level of serotonin in the brain tissue (0.0558±0.025 µg/g wet tissue) when compared with control rats (0.2826±0.033 µg/g wet tissue). It can be seen that based on the dose of SDI 3, an increase in the serotonin level (0.1434±0.038 and 0.2025±0.035 µg/g wet tissue) was noticed for low and high doses. The low dose of SDI 6 was non-significant $P > 0.05$ (0.1511±0.01 µg/g wet tissue) whereas at high dose it showed significant difference $P<0.01$ (0.2213±0.021 µg/g wet tissue) when compared with Group-II rats. The level of serotonin was high in Group VII animals treated with Selegiline (0.1989±0.034 µg/g wet tissue) when compared to rats in groups II ($P < 0.05$). (Table 31)

The data obtained in the study clearly proved the decreased in level of norepinephrine in Group-II rats (0.0977±0.013 µg/g wet tissue) and was found to be significant ($P<0.01$) when compared with control group of rats (0.3509±0.025 µg/g wet tissue). The rats treated with SDI 6 showed a dose dependent increase in norepinephrine levels at doses of 100 and 200 mg/kg (0.1968±0.008 and 0.3106±0.029 µg/g wet tissue respectively) and exhibited a significance of $P<0.01$. SDI 3 also showed a dose dependent increase in norepinephrine level, the values were found to be at non-significant in low dose (0.162±0.011 µg/g wet tissue) and significant difference in high dose (0.2648±0.015 µg/g wet tissue). The standard drug treated group also exhibited an increased level of norepinephrine with significance $P<0.01$ (0.323±0.014 µg/g wet tissue) (Table 31).

**Table 31: Effect of SDI 3 and SDI 6 on monoamine levels in the rat brain in rats exposed to CUMS**

| Group | Serotonin (µg/g wet tissue) | Nor-adrenaline (µg/g wet tissue) |
|---|---|---|
| Control | 0.2826±0.033 | 0.3509±0.025 |
| Negative control | $0.0558\pm0.025^{\#\#}$ | $0.0977\pm0.013^{\#\#}$ |
| SDI 3 – LD | $0.1434\pm0.038^{ns}$ | $0.162\pm0.011^{ns}$ |
| SDI 3 – HD | $0.2025\pm0.035^{**}$ | $0.2648\pm0.015^{**}$ |
| SDI 6 – LD | $0.1511\pm0.01^{ns}$ | $0.1968\pm0.008^{**}$ |
| SDI 6 – HD | $0.2213\pm0.021^{**}$ | $0.3106\pm0.029^{**}$ |
| Selegiline | $0.1989\pm0.034^{*}$ | $0.323\pm0.014^{**}$ |

Values were expressed in mean ± SEM.

Data were analysed by one-way ANOVA followed by Dunnett's test.

In negative control group, $^{\#\#}P < 0.01$ compared to control group. In treatment groups, **P < 0.01and $^{ns}P > 0.05$ compared to negative control group.

**Effect of SDC 1 and SDC 8 on total protein level in the rat brain**

The standard graph of protein in this study were constructed by plotting the concentration vs absorbance of the standard. $y = 0.0539x + 0.0156$ ($R^2 = 0.9787$). The level of total protein in animals was shown in Table 32. The total protein level in the CUMS alone exposed group for 42 days (783.88±22.54 mmoles/min/mg wet tissue) was found to be significantly decreased (P<0.01) when compared to the control group (4000±408.25 mmoles/min/mg wet tissue). There was a dose dependent and significant increase in the level of total protein in the rats treated with SDI 6 at doses of 100 and 200 mg/kg (2133.33±161.59 and 2733.33±261.41 mmoles/min/mg wet tissue), Selegiline also showed a rise in the total protein level (3066.66±528.63 mmoles/min/mg wet tissue) and these were found to be significant (P<0.01) when compared to the negative control (Group-II). There was also a prominent increase in the levels of total protein in the SDI 3 treated animals at low and high

dose (2066.66±187.08 and 2400±281.86 mmoles/min/mg wet tissue) and values obtained were found to be significant (P<0.01).

**Table 32: Effect of SDI 3 and SDI 6 on level of total protein in rat brain**

| Group | Total protein (mmoles/min/mg wet tissue) |
|---|---|
| Control | 4000±408.25 |
| Negative control | 783.8828±22.543## |
| SDI 3 – LD | 2066.667±187.08* |
| SDI 3 – HD | 2400±281.86** |
| SDI 6 – LD | 2133.333±161.59* |
| SDI 6 – HD | 2733.33±261.41** |
| Selegiline | 3066.667±528.63** |

Values were expressed in mean ± SEM.

Data were analysed by one-way ANOVA followed by Dunnett's test. In negative control group, ##P < 0.01 compared to control group. In treatment groups, **P < 0.01 and *P < 0.05 compared to negative control group.

**Effect of SDI 3 and SDI 6 on MAO A and MAO B enzyme levels in the rat brain**

A significant increase (P < 0.01) in MAO-A and MAO-B was found in group II (1.28±0.063 & 1.21±0.045 nmoles/min/mg protein) when compared to the group I (0.19±0.002 & 0.23±0.005 nmoles/min/mg protein). The SDI 6 significantly (P < 0.01) elevated the monoamines level in the treatment group by decreasing the MAO-A and MAO- B enzymes. At low dose, the level of MAO-A and MAO-B was found to be 0.78±0.021 & 0.66±0.025 nmoles/min/mg protein respectively. At high dose, the level of MAO-A and MAO-B enzymes was highly decreased (0.59±0.033 and 0.44±0.013 nmoles/min/mg protein respectively) when compared to negative control (1.28±0.063 & 1.21±0.045 nmoles/min/mg protein) (P < 0.01). The data clearly demonstrated that SDI 3 also exhibited dose dependent decrease in the level of MAO-A and MAO-B enzymes at high and low dose (P < 0.01). The level of MAO-A and MAO-

B enzyme was less in Group- VII animals treated with Selegiline (0.37±0.038 and 0.44±0.015 nmoles/min/mg protein respectively) when compared to rats in groups II (Table 33).

**Table 33: Effect of SDI 3 and SDI 6 on level of monoamine oxidase-A and monoamine oxidase-B in rat brain**

| Group | MAO-A (nmoles/min/mg protein) | MAO-B (nmoles/min/mg protein) |
|---|---|---|
| Control | 0.193±0.0026 | 0.239±0.0053 |
| Negative control | 1.287±0.0632$^{\#\#}$ | 1.219±0.0459$^{\#\#}$ |
| SDI 3 – LD | 0.644±0.0533$^{**}$ | 0.726±0.0062$^{**}$ |
| SDI 3 – HD | 0.383±0.0111$^{**}$ | 0.613±0.0367$^{**}$ |
| SDI 6 – LD | 0.783±0.021$^{**}$ | 0.661±0.0251$^{**}$ |
| SDI 6 – HD | 0.593±0.0334$^{**}$ | 0.445±0.0138$^{**}$ |
| Selegiline | 0.377±0.038$^{**}$ | 0.444±0.0158$^{**}$ |

Values were expressed in mean ± SEM.

Data were analysed by one-way ANOVA followed by Dunnett's test.

In negative control group, $^{\#\#}P < 0.01$ compared to control group. In treatment groups, **P< 0.01 compared to negative control group.

**Effect of SDI 3 and SDI 6 on level of antioxidants and Lipid peroxidation in rat brain**

The level of superoxide dismutase (SOD) in Group-II rats (12.78±1.66 nmoles/min/mg protein) was found to be significant decreased (P<0.01) when compared with control group (54.06±4.77 nmoles/min/mg protein). The Group III and IV treated with SDI 3 at doses of 100 and 200 mg/kg depicted a dose dependent increase in the SOD levels (28.07±5.48 and 41.57±5.3 nmoles/min/mg protein respectively), the standard Selegiline also showed a significant (P<0.01) increase in the level of SOD (46.28±4.18 nmoles/min/mg protein) when compared to negative control rats. SDI 6 at low and high dose also showed a gradual increase based on dose in the SOD level (30.58±1.8 and 43.33±3.28 nmoles/min/mg protein) and was statistically significant (P<0.01) (Table 34).

Catalase (CAT) level in Group II (14.03±0.905 µmol/min/mg protein) was found to be significantly (P<0.01) decreased when compared to control group of rats (38.44±3.003 µmol/min/mg protein). Administration of SDI 3 to group-III and IV exhibited a dose dependent increase in the levels of CAT (22.15±3.96 and 30.21±3.61 µmol/min/mg protein) and was found to be significant with P<0.01. The CAT levels in Group-V and VI treated with SDI 6 at low and high dose have shown significant (P<0.01) increase in the level of CAT (20.34±1.19 and 33.93±2.46 µmol/min/mg protein). The Group V standard also exerted a significant (P<0.05) increase in the level of CAT (35.156±3.24 µmol/min/mg protein) (Table 34).

The levels of glutathione peroxidase (GPx) in Group-II rats (23.65±5.36 nmol/min/mg protein was found to be significantly (P<0.01) decreased when compared to control (40.36±6.55 nmol/min/mg protein). Group III and IV treated with SDI 3 at low (P> 0.05) and high dose (P < 0.05) for 42 days exhibited a dose dependent increase in the levels of GPx (27.76±6.95 and 34.58±7.39 nmol/min/mg protein). The SDI 6 treated group exerted significant increase in level of GPx 28.82±4.53 and 35.45±5.06 nmol/min/mg protein at low and high dose respectively. The standard drug, Selegiline treated group (P < 0.05) also exhibited an increased level of GPx (36.34±6.88 nmol/min/mg protein) (Table 34).

The data obtained in the study clearly showed decreased level of glutathione reductase (GSSH) in the Group-II rats (10.69±1.34 nmol/min/mg protein) and was found to be significant (P<0.01) when compared with control group of rats (34.44±4.60 nmol/min/mg protein). The rats treated with SDI 3 showed a dose dependent increase in GSSH levels at doses of 100 and 200 mg/kg (21.07±2.84 and 31.31±5.43 nmol/min/mg protein) exhibited a significance of P<0.01. The SDI 6 also showed a significant increase in the level of GSSH at low and high dose (17.75±1.58 and 32.40±1.41 nmol/min/mg protein). Treatment with standard drug also increased the level of antioxidant GSSH level (30.57±4.50 nmol/min/mg protein) (Table 34).

The levels of reduced glutathione (GSH) in Group-II (19.13±1.54 nmol/min/mg protein) was compared with the control group (39.95±1.52 nmol/min/mg protein) and results were found to be significant (P<0.01). Increase in the level of GSH was observed in rats treated with SDI 3 low dose and high dose (21.79±1.73 and 29.86±1.89 nmol/min/mg protein) and was statistically significant. The SDI 6 treated group showed a dose dependant increase in the GSH level at 23.73±1.85 nmol/min/mg protein for low dose and 33.79±1.64 nmol/min/mg protein for high dose. The group treated with standard drug Selegiline also exhibited a better

increase in the level of GSH (37.6±1.13 nmol/min/mg protein) with level of significance of P<0.0 (Table 34).

The data obtained in the study clearly proved the increased levels of Lipid peroxidation (MDA) in the Group-II rats (7.70±0.24 nmol/min/mg protein) and was found to be significant (P<0.01) when compared with control group of rats (2.46±0.03 nmol/min/mg protein). The rats treated with SDI 3 showed a dose dependent decrease in MDA levels at doses of 100 and 200 mg/kg (4.19±0.06 and 2.69±0.025 nmol/min/mg protein) exhibited a significance of P<0.01. The SDI 6 also showed a significant decrease in the level of MDA at low and high dose (3.74±0.10 and 2.60±0.04 nmol/min/mg protein). Treatment with standard drug, selegiline also decreased the level of MDA (2.76±0.041 nmol/min/mg protein) (Table 34).

**Table 34: Effect of SDI 3 and SDI 6 on level of enzymatic and non-enzymatic antioxidants in brain**

| Group | Enzymatic antioxidants | | | | Non-enzymatic antioxidant | Lipid Peroxidation |
|---|---|---|---|---|---|---|
| | Superoxide dismutase (nmoles/min/mg protein) | Catalase (µmoles/min/mg protein) | Glutathione proxidase (nmoles/min/mg protein) | Glutathione reductase (nmoles/min/mg protein) | Reduced glutathione (nmoles/min/mg protein) | MDA (nmoles/min/mg protein) |
| Control | 54.06±4.77 | 38.44±3.003 | 40.36±6.55 | 34.44±4.60 | 39.95±1.52 | 2.46±0.035 |
| Negative control | 12.78±1.66$^{\#\#}$ | 14.03±0.9056$^{\#\#}$ | 23.65±5.36$^{\#\#}$ | 10.69±1.34$^{\#\#}$ | 19.13±1.54$^{\#\#}$ | 7.70±0.24$^{\#\#}$ |
| SDI 3 – LD | 28.07±5.48$^{ns}$ | 22.15±3.967$^{*}$ | 27.76±6.95$^{ns}$ | 21.07±2.84$^{**}$ | 21.79±1.73$^{ns}$ | 4.19±0.064$^{**}$ |
| SDI 3 – HD | 41.57±5.3$^{*}$ | 30.21±3.616$^{**}$ | 34.58±7.39$^{*}$ | 31.31±5.43$^{**}$ | 29.86±1.89$^{**}$ | 2.69±0.025$^{**}$ |
| SDI 6 – LD | 30.58±1.8$^{**}$ | 20.34±1.192$^{ns}$ | 28.82±4.538$^{ns}$ | 17.75±1.58$^{*}$ | 23.73±1.85$^{ns}$ | 3.74±0.10$^{**}$ |
| SDI 6 – HD | 43.33±3.28$^{**}$ | 33.93±2.464$^{**}$ | 35.45±5.06$^{*}$ | 32.40±1.41$^{**}$ | 33.79±1.64$^{**}$ | 2.60±0.044$^{**}$ |
| Selegiline | 46.28±4.18$^{**}$ | 35.56±3.248$^{**}$ | 36.34±6.88$^{*}$ | 30.57±4.50$^{**}$ | 37.60±1.13$^{**}$ | 2.76±0.041$^{**}$ |

Values were expressed in mean ± SEM. Data were analysed by one-way ANOVA followed by Dunnett's test. In negative control group, $^{\#\#}P < 0.01$ compared to control group. In treatment groups, $^{**}P < 0.01$, $^{*}P < 0.05$ and $^{ns}P > 0.05$ compared to negative control group.

# DISCUSSION

Depression is one of the major mood disorders, characterized by a combination of symptoms that interfere with a person's ability to work, sleep, study, eat and enjoy once-pleasurable activities and prevents a person from functioning normally. Known causes of depression do not provide sufficient explanation of pathophysiology, despite extensive research in this area. It is believed that the process is multifactorial and has many subtypes with more than one etiology. Depression is an important global public health problem due to both its relatively high lifetime prevalence and the significant disability that it causes. The demand for curing depression and other mental health conditions is on the rise globally.[101]

WHO stated that the depression is the fourth leading cause of disease worldwide. It affects the individual's socioeconomic status, family life, and, therefore, need medical attention. [1] At a societal level, it leads to loss of productivity and economic burden. Though effective treatments are available, it continues to be an under-recognized and undertreated disorder. The average age of onset for major depression is 24 years as per the recent epidemiological research, though it can begin at any time throughout the lifespan. One of the consistent findings across almost all research studies reveals that women are twice as likely to have depression compared to men. [102]

Isatin is an endogenous compound isolated in 1988 and reported to possess a wide range of central nervous system activities. Isatin is a heterocyclic moiety which can be used for the synthesis of a large variety of heterocyclic compounds such as quinolines, indoles, and as raw material for medicinal important drugs. Isatin and its derivatives were reported to possess pro-convulsant and anti-convulsant activities. Isatin derivatives also displayed potent antidepressant activities and possess CNS-monoamine oxidase (MAO) inhibition. Schiff bases and Mannich bases of isatin were reported to possess anti-bacterial, anti-fungal, anti-viral, anti-HIV, anti-protozoal, and anti-helminthic activities. [103]

In *in silico* docking studies, the Lipinski parameters such as molecular weight, Log P, number of hydrogen bond donors, number of hydrogen bond acceptors and violations in these parameters were screened for the different synthetic derivatives of isatin (SDI 1 to SDI 50). It was found that among the 50 compounds, 10 compounds showed violations i.e., molecular weight was greater than 500 Daltons and Log P was greater than 5. Hence these compounds were eliminated from the study.

The predicted bioactivities of the different synthetic derivatives of isatin (SDI 1 to

SDI 50) was within a range of considerable bioactive to bio inactive (bioactive score more than 0.00 is found to possess considerable biological activities, while values -0.50 to 0.00 are expected to be moderately active and if score is less than -0.50 it is presumed to be bio inactive). Considerable to moderate GPCR ligand action were observed with the SDI compounds within a range of -0.01 to -0.71. The 10 compounds which were considered as violating Lipinski rule of 5 was also considered bio inactive. The remaining 40 compounds were selected and further evaluated for ADMET studies.

The primary criteria that was followed in ADMET property of the selected SDI compounds was to determine the blood brain barrier penetration since the antidepressants work by acting on the brain to exert its therapeutic action. Hence in this study preADMET online tool was used to access the BBB penetration and pharmacokinetic properties. The 40 compounds that passed the test in the drug likeliness parameters were subjected to evaluation of ADMET properties. [104]

The compounds which has shown the value more than 1.0 in the BBB penetration were considered as having ability to cross blood brain barrier. In preADMET studies, out of 40 compounds, 6 compounds have proven BBB penetration value more than 1.0 and they were considered as lead compound for next *in silico* study. The selected 6 compounds were also having good pharmacokinetic profile. [104]

After completion of preADMET, next step of molecular docking study was performed. It was used to evaluate the binding energy and inhibitory potential of the selected lead compound against targeted enzyme (MAO- A and IDO1). If the lead compound required low binding energy and lower intermolecular energy for inhibiting the targeted enzyme, then the lead compound may be considered as having higher inhibitory potential against the targeted enzyme.

AutoDock was run several times to get various rigid docked conformations, and used to analyse the predicted docking energy. The binding sites for these molecules were selected based on the ligand-binding pocket of the templates. AutoDock Tools provide various methods to analyse the results of docking simulations such as, conformational similarity, visualizing the binding site and its energy and other parameters like intermolecular energy and inhibition constant. For each ligand, best docking poses were generated and scored using AutoDock 4.2 scoring functions. Analysis of the receptor/ligand complex models generated after successful docking of the SDI compounds was based on the parameters such as hydrogen bond interactions, π – π interactions, binding energy of active site residues and orientation of the docked compound within the active site. As a general rule, in most of the

potent anti-depressant compounds, both hydrogen bond and π – π hydrophobic interactions between the compound and the active sites of the receptor have been found to be responsible for mediating the biological activity. [105]

The SDI compounds which exhibited better BBB penetration were docked against monoamine oxidase and indoleamine 2,3-dioxygenase. Based on binding energy, inhibitory constant and intermolecular energy, two compounds which showed higher and lower binding energy were selected for *in vitro* studies. Based on these perspective, the compound SDI 3 and SDI 6 showed lower and higher binding energy respectively and was selected for *in vitro* studies.

The *in-vitro* MAO inhibitory activity of both synthetic derivatives of isatin and standard Selegiline was evaluated by measuring its MAO-A inhibitory activity. The synthetic derivatives of isatin showed significant MAO-A inhibitory activity at varying concentrations. There was a dose dependent increase in the percentage inhibition for all the concentrations. The study clearly established that SDI 6 had better inhibitory potential than SDI 3 when compared with the standard drug. Based on the *in vitro* results, the two compounds were evaluated for *in vivo* studies. [106]

The acute toxicity study was conducted for compound SDI 3 and SDI 6, the results showed that the compounds did not show any significant morbidity and mortality or any clinical symptoms of toxicity at the maximum dose tested i.e., its $LD_{50}$ value more than 2000 mg/kg. The live phase observations were also seen to be normal. Two dose levels were used to ascertain any dose dependent increase in the protection shown by SDI compounds.

The experimental design consisted of the use of rats under control (CMC alone); negative control (CUMS alone); positive control (CUMS and Selegiline); two test groups SDI 3 and SDI 6 with two dose levels along with CUMS were used.

Chronic stress is used in animal models to induce behavioural, physiological and neural changes relative to human depression. The CUMS model is an acclaimed and authentic animal model of depression with high translational potential since this model can mimic the core symptoms of the depression by exposing animals to diverse kind of unpredictable stress every day. This model is widely used for studying antidepressant candidates because of its reliability. The isolation of animals done in this study is relevant to several neuropsychiatric disorders, such as schizophrenia, anxiety and depression. The unpredictable mixture of (psycho) social and physical stressors not only reduces the chance of adaptation but also better mimics the variability of stressors encountered in daily life.

After 6 weeks of chronic mild stress, the animals have shown a reduced preference to consume sucrose solution, suggesting depression- like effects. [107,108]

The tail suspension and forced swimming tests were two behavioural tests in a rodent that predicted the clinical efficacy of many types of antidepressant treatments. Porsolt *et al.*, (1978) described an animal model for assessing the effect of antidepressant drugs. This test was based on behavioural despair, i.e. the rat after placing in water become immobile and float with stretched limbs which are an indication of depression. Drugs which reduced the period of immobility belonged to the group of antidepressants. The Porsolt test is an extensively used, validated model and included in the battery of test for screening drugs having antidepressant-like activity. The increased immobility time observed in the study for rats in all groups indicated the occurrence of depression. Treatment with respective drugs decreased immobility time at week 4 to 6 indicating the effectiveness of drug in treatment of depression. The test compound SDI 6 was observed to decrease the immobility time better than SDI 3. This proved that halogen substituted isatin derivatives had better efficiency in the treatment of depression. [109,110]

The tail suspension test is a well-characterized behavioural model that thinks to be predictive of depressant activity in human. In the tail suspension test, animals subjected to inescapable, aversive situation showed agitation and immobility which is an indication of depression. When rats were suspended by the tail they were subjected to short-term inescapable stress and they adopt an immobile posture. The increase in such activity (i.e. the decrease in immobility) in the TST is strongly correlated with antidepressant effects in humans. The CUMS exposed animals showed an increase in immobility time indicating that the animals were in depressed state. The treatment with SDI compounds significantly reversed the increase in immobility time showing the potential of SDI compounds having antidepressant activity. [111]

Anxiolytic compounds selectively increase the percentage of time spent into the open arms and, in contrast, anxiety produced in rats by CUMS selectively decrease the percentage of time spent into the open arm and increase the time spent in the closed arm. Two doses of SDI also showed proportional increase in reversal of change in parameters.

A marked reduced interest or pleasure in activities previously considered pleasurable is a main symptom in mood disorder. This condition is called “anhedonia” and term refers to feel pleasure that is one complex phenomenon of processing and responding to reward. The theoretical rationale in the CUMS model is that it simulates anhedonia, a loss of responsiveness to pleasant events, which is a core symptom of depression. CUMS causes a

decrease in responsiveness to rewards in a variety of different behavioural paradigms (consumption of sweet diets - sucrose). While each of these behavioural changes is susceptible of a variety of interpretations, the most parsimonious account is that CUMS causes a generalized decrease in sensitivity to rewards (anhedonia). In the sucrose preference test, rats were given access to a choice between 1% sucrose solution and drinking water. The CUMS exposed rats showed decrease in sucrose preference indicating that the animals were in loss of responsiveness towards reward. Whereas upon treatment with the test compounds significantly reversed the loss of responsiveness i.e., animals were more likely towards rewards. SDI 6 treated animals showed better reversal of anhedonia effect and the results were found to be significant as that of standard Selegiline. [112]

In stressful situations, various inputs of the hypothalamus like the cerebral cortex, limbic system, visceral organs etc. pass the information about stress onto the hypothalamus which results in the activation of the hypothalamic pituitary adrenal pathway. The HPA axis hyperactivity is a risk factor for the onset of major depression and overexposure of the brain to corticosteroid hormones indeed results in changes in the structure and function of the brain. Activation of the HPA axis after exposure to a brief stressor results in enhanced level of circulating corticosteroid hormones, which on the one hand helps to restrain the hormone release via negative feedback at the level of the paraventricular nucleus of hypothalamus.[112-113]

High concentration of corticosterone is released during stressful situations in rats and this may be due to dysfunction of negative feedback mechanism. Hence increased concentration of corticosterone in the serum is an indication of stress. The experiments conducted as a part of this study showed significantly increased corticosterone levels ($P < 0.01$) in negative control group compared to the control group. During treatment with SDI 6, the level of corticosterone was decreased, this showed the anti-stress potential of the SDI 6. The compound SDI 3 decreased corticosterone level but the decrease was not that produced by SDI 6. The increased corticosterone level is considered as biomarker of depression, test compounds decreased corticosterone providing the efficient treatment of depression. [113]

IDO is a tryptophan-degrading enzyme whose increased activity may lead to increase in the formation of kynurenine due to tryptophan metabolism, resulting in the decrease of 5-HT synthesis. Therefore, the abnormal expression of tryptophan and IDO may cause the dysfunction of the 5-HT system, leading to the occurrence of mental disorders such as depression. The metabolites of kynurenine pathway (3- hydroxy-kynurenine)

presented might have influence on the metabolism of neurotransmitters and generate a large amount of oxygen free radicals to enhance the activity of monoamine oxidase and accelerate the metabolism of 5-HT in synaptic cleft. Hippocampus is rich in neurotransmitters (5-HT, DA and NE) and receptors and is closely related to emotion and it is the regulating centre for stress response, and its structure and function could be changed by chronic stress. Hippocampus is also a target of stress hormones, and stress and high glucocorticoids can affect the hippocampal neuroplasticity. [114]

The first neurochemical theory of depression was monoamine hypothesis which postulated that the major cause of depression is the decrease or deficiency of biogenic amine function i.e. imbalance of available monoamines- serotonin, norepinephrine and this results in disturbed synthesis, storage and release of monoamines or subnormal monoamine receptors functioning in certain regions of the brain**.** The monoamines play a very important role in the etiopathogenesis of depression. The spontaneous and experimentally induced deficiencies in monoamines are well documented and implicated in the onset of depression. The clinically used antidepressant molecules that have been developed in the past were aimed to increase extracellular level of biogenic amines 5 HT and NE within the brain either by blocking the reuptake or inhibiting monoamine oxidase which causes degradation of monoamines**.** [113,114]

Serotonin and norepinephrine, which have been implicated in the pathophysiology of depression are removed from the synaptic cleft by sodium-dependent uptake through transporters located in the plasma membrane of presynaptic cells, in close association with $Na^+$, $K^+$- ATPase activity. In the present study, rats treated for 42 days with SDI 3/SDI 6/Selegiline showed improvement in the levels of 5-HT and NE in the brain. It is also observed that SDI 6 at 200 mg/kg significantly increased serotonin and norepinephrine levels indicating the decreased activity of Na-K-ATPase enzyme leading to reduced uptake through transporters. [115]

Monoamine oxidase is a mitochondrial enzyme which catalyses the oxidative deamination of a variety of monoamines such as serotonin and norepinephrine. The pathophysiology of depression involves the abnormal activity of the enzyme and leads to dysfunction in monoaminergic neurotransmission in central nervous system. The important target for treatment of depression is to inhibit monoamine oxidase, thereby producing antidepressant activity. Inhibition of selective MAO-A leads to increase in serotonin and norepinephrine levels in the brain. A simple and sensitive spectrophotometric method for determination of monoamine oxidase activity was used by many researchers to study the

MAO inhibitory activity. [115]

In the present investigation, it has been demonstrated that the SDI 6 significantly inhibited *in vivo* MAO-A activity in rats in a dose-dependent manner. These findings suggested that anti-depressant effects of SDI 6 in animal models of immobility tests may be related to the inhibitory activity of MAO, especially to that of MAO-A.

The enzyme monoamine oxidase B is mainly involved in the metabolism of dopamine. The inhibition of MAO- B helps in increasing the concentration of dopamine leading to alleviation of symptoms in Parkinson's disease. The study results have shown that there was a significant decrease in the levels of MAO- B, indicating that SDI 3 & SDI 6 may also be considered as potent antiparkinsonian agent.

Oxidative phosphorylation, which takes place in the mitochondria of the cell, is the major source of ATP in aerobic organisms. The downside of this important process is that it often produces free radicals, commonly resulting in elevated levels of ROS and RNS. The brain has one of the highest mass-specific oxygen consumption rates in the body, even the smallest imbalances in antioxidant defence mechanisms and oxidative stress can be deleterious to neurons. Oxidative stress related molecular mechanisms may represent one factor influencing the etiology of depressive and anxiety disorders. An imbalance between the generation of ROS by endogenous/ exogenous pro-oxidants and the defence mechanism against ROS by antioxidants has been found in these disorders. [116]

It is extensively reported that CUMS impairs the antioxidant status of brain tissue, presumably by generating excessive ROS and increasing oxidative stress. Some brain regions, especially those in the limbic system (e.g. hippocampus and frontal cortex), act in concert to mediate the symptoms of depression and anxiety, and seem to be strongly affected by the deleterious effects of an oxidative insult, making them excellent candidates to observe antioxidant factors. Decreased expression of Cu/Zn SOD in the hippocampus is often seen in stressed animals.[120] Treatment with antidepressants restores Cu/Zn SOD expression in a dose dependent manner. The findings also relate that stressed animals exhibited a decrease in antioxidant level in brain whereas the treatment with SDI compounds restored antioxidant levels in dose dependent manner. SDI 6 showed a better restoration level of antioxidants when compared to SDI 3 and the standard drug Selegiline and this may be due to reduced generation of ROS and occurrence of oxidative stress.

The current study revealed an increase in lipid peroxidation in the prefrontal cortex of rats exposed to stress, which indicates oxidative stress. This oxidative stress causes depression which is accompanied by an increase of lipid peroxidation in the brain. Highly

reactive oxygen metabolites act on unsaturated fatty acids of phospholipid components of membranes to produce malondialdehyde, a lipid peroxidation product. Reactivity of MDA may cause damage to DNA. In the brain, there is a significant amount of unsaturated fatty acids, which are susceptible to peroxidation. Moreover, in the brain, there is a relatively poor anti-oxidant defense system. This creates a risk of DNA damage and disturbances in secondary electron transport and cellular damage. [118,119] Lipid peroxidation decreases the life span of neurons, affects neurotransmitter release, and was reported as a major contributor to the loss of cell function under stress conditions in depression. Treatment with SDI derivatives have reduced the production of malondialdehyde in a significant manner and in the present study SDI 3 showed good reduction of malondialdehyde when compared with SDI 6.

## CONCLUSION

This study highlights the possible interaction between stress and immune-inflammatory pathways in the pathogenesis of depression and suggests an animal model that addresses these pathways. The molecular docking with AutoDock 4.2 using the enzymes monoamine oxidase and indoleamine 2,3-dioxygenase for the selected compounds of synthetic derivatives of isatin resulted in excellent inhibitory potential when compared to that of standard. Based on the results of docking studies synthetic derivatives of isatin 3 and 6 were selected and evaluated for *in vitro* studies and tested at two dose levels (100 and 200 mg/kg) on experimental rats induced with chronic unpredictable mild stress depression. The test compounds exerted good antidepressant effect in rats. Treatment with SDI 3 and SDI 6 augmented the endogenous neurotransmitters (serotonin and norepinephrine), significantly restoring most of the altered behavioural parameters and established protective effects against oxidative stress caused by free radicals.

Chronic unpredictable mild stress stimulates hypothalamus-pituitary adrenal axis and enhances the release of corticosterone contributing to stress-induced depression in rats. Chronic unpredictable mild stress augmented the production of indoleamine 2,3-dioxygenase enzyme and resulted in increased production of kynurenine thereby stimulating the formation of free radicals with reduced synthesis of serotonin producing depression. Treatment with SDI 3 and SDI 6 decreased corticosterone level, blocked indoleamine 2,3-dioxygenase and effectively reduced kynurenine production and increase the formation of serotonin. Synthetic derivatives of isatin 3 and 6 inhibited formation of monoamine oxidase-A and decreased the metabolism of serotonin and increased the availability of serotonin in hippocampal and prefrontal regions in brain of rat and prevented depression. Since, the synthetic derivatives of isatin 3 and 6 inhibited monoamine oxidase-B it might also have the potential to treat neurodegeneration disease like Parkinson's.

In conclusion, the study suggested that synthetic derivatives of isatin 3 and 6 compounds possess antidepressant and antioxidant properties, which might be helpful in preventing stress induced depression and the related neurodegenerative diseases as a result of imbalance of monoamines. Molecular level studies and further *in vitro* and *in vivo* models are required to substantiate this antidepressant activity and to corroborate the results of synthetic derivatives of isatin 3 and 6 before these compounds can be taken for further studies in human volunteers.

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# ANNEXURES

## INSTITUTIONAL ANIMAL ETHICS COMMITTEE

(CPSCEA Registration # 1559/PO/Re/S/11/CPCSEA)

**College of Pharmacy**

Sri Ramakrishna Institute of Paramedical Sciences

**(Educational Service of M/s SNR Sons Charitable Trust)**

Coimbatore – 641 044.

## IAEC PROTOCOL APPROVAL CERTIFICATE

Date: 14/09/2019

**Approval #:1559/PO/Re/S/11/CPCSEA**

**IAEC PROTOCOL#: COPSRIPMS/IAEC/PG/Pharmacology/001/2019-2020**

**IAEC PROTOCOL TITLE:** Treatment of depression in rats exposed to chronic unpredictable mild stress using synthetic derivatives of Isatin

Dear Dr. K. Asok Kumar,

This is to certify that above mentioned animal study protocol has been approved in IAEC meeting held on 14/09/2019 with following conditions:

PI : Mr. Ajin P Kurian

Duration of Study : months/years (From 14/09/2019 to13/09/2020)

Animal Sanctioned : 60 *Wistar* Rats

Species : Rats

Strain : *Wistar*

Sex/Age : Male (42 Rats) and Female (18 Rats) / 12 Weeks

Total No. : 60

It is requested to get prior approval of IAEC in case of any deviation/changes in submitted protocol. Please maintain the Form D & provide the photocopy to IAEC along with project report at defined interval.

| | | |
|---|---|---|
| **Member Secretary** | **Chairman** | **Main nominee** |
| **IAEC, COP, SRIPMS** | **IAEC, COP, SRIPMS** | **CPCSEA** |
| | | CG. ARIHARASIVAKUMAR |