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Research Protocol

Pharmacology Section

Assessment of Antidepressant Activity of Ethanolic Extract of *Hibiscus rosa sinensis* Linn. Petals in Comparison with Fluoxetine and its Toxicity in Swiss Albino Mice: A Preclinical Research Protocol

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ABSTRACT

Introduction: Depression is the most common mental health condition today, with about 5% of adults worldwide thought to be affected. *Hibiscus rosa sinensis* (HRS) Linn (Malvaceae) blooms are a rich source of flavonoids, which have been shown to possess antidepressant properties. The current investigation seeks to evaluate the antidepressant effects of flavonoids in HRS Linn petals.

Need of the study: Strong allopathic drugs such as monoamine oxidase inhibitors, Selective Serotonin Reuptake Inhibitors (SSRIs) and tricyclic antidepressants increase the levels of these neurotransmitters. However, there are disadvantages associated with these medications. Since there are not many research studies on antidepressants derived from plants, this topic requires assessment. This approach offers a gradual and effective healing process, utilising harmless herbs and derived formulations that are also cost-effective and reliable.

Aim: To compare the safety and effectiveness of ethanolic extracts of HRS Linn petals to fluoxetine (an SSRI) in male Swiss albino mice.

Materials and Methods: An experimental study will be conducted at the Animal House, Datta Meghe Institute of Higher Education and Research (DMIHER), Sawangi, Wardha, Maharashtra, India from August 2024 to February 2025. The study procedure will involve procuring standard drugs, screening animals, preparing a solution for the test drugs and determining the LD50. The antidepressant effects of crude ethanol extract from the floral parts of HRS will be compared to standard fluoxetine treatment using four different behavioural tests: the Elevated Plus Maze (EPM), tail suspension, forced swim and Open Field Tests (OFT). The extract will be administered at doses of 100 mg/kg, 250 mg/kg and 500 mg/kg, while fluoxetine will be given at a dose of 15 mg/kg. The acute toxicity of the HRS extract will also be assessed using four different doses: 5 mL/kg, 50 mg/kg, 300 mg/kg and 2000 mg/kg. The toxicity test will be evaluated based on Organisation for Economic Co-operation and Development (OECD) 423 guidelines. An Analysis of Variance (ANOVA) test will be applied for intergroup comparison of differences in behavioural tests and acute toxicity among these four groups. A p-value <0.05 will be considered significant.

Keywords: Depression, Elevated plus maze test, Forced-induced swimming test, Open field test, Tail suspension test

INTRODUCTION

According to a poll by the World Health Organisation (WHO), 280 million people worldwide experience depression. Its defining characteristics include a persistent depressive episode, loss of enjoyment and lack of interest in activities. Numerous factors, such as relationships, stress, heavy educational loads and economic problems, can aggravate depression. Globally, 5.7% of adults over 60 and 5% of adults in general (4% of men and 6% of women) experience depression [1]. It is primarily caused by a deficiency of certain neurotransmitters, namely glutamate, serotonin, nor epinephrine and gamma-aminobutyric acid. Among the psychological therapies that work well for depression are behavioural activation, interpersonal psychotherapy, cognitive behavioural therapy, problem-solving therapy and SSRIs like fluoxetine, which are antidepressant drugs [2]. In low- and middle-income nations, over 75% of individuals do not receive treatment, even though there are established and effective treatments for mental disorders. This lack of consideration is impeded by the stigma society places on psychological maladjustment, a lack of support for mental health treatments and a shortage of therapeutic professionals [3-5].

The HRS, commonly known as the China rose, is a member of the Malvaceae family. Due to its therapeutic properties, various parts of this plant have been utilised in traditional medicine for a long time. It is grown throughout India and originated in tropical Asia. The plant produces five crimson petals on its pediculate blooms. Typically, the leaves are simple, elliptic and green, with a capsular structure on the fruits. It contains a variety of chemical substances, including cyclopropanoids, malate, beta-rosasterol, anthocyanin, methyl sterculate, methyl-2-hydroxysterculate, 2-hydroxysterculate, amino acids (aspartic acid and asparagine), fats, calcium, phosphorus, iron, thiamine, riboflavin, niacin, flavonoids, fibers and vitamin C. This herb is used to treat cardiac issues and lung conditions. In addition to its high nutritional value, it possesses emmenagogue, aphrodisiac, emollient and abortifacient properties [6,7].

Depression is common, affecting more females than males. It can lead to suicide, with women being affected about 50% more often than men. Suicidal thoughts may occur in some individuals and depression is believed to negatively affect cognitive behaviour. Each year, almost 700,000 people take their own lives. According to the WHO, as of March 31, 2023, the leading causes of death among individuals aged 15 to 29 are mental health disorders, moderate to severe depression and suicide [8]. Individuals suffering from depression may notice an improvement in mood with antidepressant medications. Almost all antidepressants affect the brain's monoaminergic transmission in some way and many of them also have other related properties [9].

The most frequent side-effects include gastrointestinal issues, which tend to improve over time as tolerance develops. However, since SSRIs activate the 5-Hydroxytryptamine type 3 (5-HT3) receptor, they often cause nausea. Loose stools can result from the inhibition of intestinal 5-HT uptake and the stimulation of 5-HT receptors on enteric plexus neurons. While SSRIs typically do not lead to weight gain, they commonly cause problems with ejaculation and orgasm, as well as headaches, anxiety, restlessness, insomnia, anorexia and dyskinesia. Reports indicate a higher frequency of epistaxis and ecchymosis, likely due to platelet dysfunction [10,11]. Additionally, SSRIs may exacerbate gastric blood loss caused by Non Steroidal Anti-Inflammatory Drugs (NSAIDs). It is known that flavonoids, such as quercetin and anthocyanin, are present in these flowers and show antidepressant effects. Hibiscus flowers may also have an impact on monoamines [12].

The present study aims to compare the safety and effectiveness of the ethanolic extract of HRS Linn petals to fluoxetine (SSRIs) in male Swiss albino mice.

Primary objectives:

- To evaluate the antidepressant activity of fluoxetine.
- To assess the effectiveness of the ethanolic extracts of petals of HRS Linn petals in mice using the appropriate screening methods.

Secondary objectives:

- To compare the antidepressant activity of HRS Linn petals with that of fluoxetine in mice using appropriate screening methods.

Null hypothesis: There will be no significant difference in the antidepressant activity of the ethanolic extract of HRS Linn petals in mice compared to fluoxetine and its toxicity.

Alternate hypothesis: There will be a significant difference in the antidepressant activity of the ethanolic extract of HRS Linn petals in mice compared to fluoxetine and its toxicity.

REVIEW OF LITERATURE

Around the world, depressive disorders are prevalent and characterised by a range of symptoms such as anhedonia, melancholy, guilt and suicidality. The current state of inadequate pharmaceutical therapy for depression contributes to the significant health burden associated with the condition. Animal models of depression have demonstrated several traits, some of which have been ameliorated by antidepressant treatment. Examples of these traits include anhedonia, helplessness, behavioural despair and neuro-vegetative changes, including irregular feeding and sleeping habits. This depression model is a quantitative assessment that mimics a manufactured sense of failure or hopelessness. This aspect of mammalian behaviour is likely more physiological than detrimental. The concept of persistent moderate stress is based on animal exposure, usually involving rats [13].

Research on various plants has demonstrated antidepressant qualities, such as ginseng (*Panax ginseng*), chamomile (*Matricaria recutita*) and *Curcuma longa* [14-16]. Khalid L et al., investigated the antidepressant efficacy of HRS Linn ethanolic extract at dosages of 100, 250 and 500 mg/kg, respectively, utilising three parameters: the Tail Suspension Test (TST), the OFT and the Forced Swim Test (FST). The usual dosage of fluoxetine was 15 mg/kg of body weight. While there were notable dose-dependent reductions in immobility time in the FST and TST, none of the three HRS dosages had any effect in the OFT. MAO "A" and "B" activity was measured using HRS extract. The findings demonstrated that this plant significantly affected MAO "A" at every dose, while only the 250 mg/kg dosage significantly affected MAO "B" [7].

Similarly, Shewale PB et al., conducted a study to assess the antidepressant-like activity of the anthocyanidins extracted from HRS flowers in the TST and FST. Like imipramine (10 mg/kg i.p.), the positive control, the ongoing review showed a significant reduction in immobility time in the TST and FST. P-chlorophenylalanine (100 mg/kg, i.p., for 3 days; an inhibitor of serotonin synthesis), prazosin (62.5 mg/kg, i.p.) and haloperidol (50 mg/kg, i.p., a classical D(2)-like dopamine receptor antagonist) all caused significant immobility in the TST and FST, respectively. MHR (methanolic extract containing anthocyanidins) and AHR (anthocyanidins) have been hypothesised to have potential energising effects (through dopaminergic, noradrenergic and serotonergic components) and provide evidence primarily at preclinical levels in the treatment of Central Nervous System (CNS) issues [17].

MATERIALS AND METHODS

An experimental study will be conducted at the Animal House of Datta Meghe Institute of Higher Education and Research, Sawangi, Wardha, Maharashtra, from August 2024 to February 2025. Ethical approval has been obtained from the Animal Ethics Committee, with the approval number DMIHER/IAEC/24-25/09.

Inclusion criteria: A total of 40 Swiss albino mice, aged 30-35 days, all males and weighing between 25-30 g, will be included in the study.

Exclusion criteria: Unhealthy or diseased mice, as well as pregnant females, will be excluded from the study.

Distribution: The mice will be divided into five groups, with six mice in each group for behavioural experiments. Before the start of the trial, the mice will be acclimatised. For acute toxicity testing, the mice will be divided into four groups, containing 10 mice in each group [18].

Selection of species and strain, health and genetic screening, anxiety screening and baseline behaviour assessment of the Swiss albino mice will be performed before the experiment. The Rota rod, triple horizontal bar, parallel bar and static rod tests will be used to evaluate the motor function and co-ordination of the mice prior to the experiment.

Rota rod: In this test, an animal, typically a rodent, is placed on a rotating rod. To avoid falling off, the animal must advance. This test is frequently used to assess balance and motor co-ordination.

Triple horizontal bar: In this method, the animal will traverse a series of horizontal bars, testing its strength and co-ordination, especially in the forelimbs.

Parallel bar: In this test, the animals navigate between parallel bars, evaluating their ability to balance and coordinate their movements.

Static rod: This method assesses the animal's balance and co-ordination by placing it on a stationary rod without the added challenge of movement [19].

Acclimatisation: The mice will be allowed to rest for one week to acclimatise to the new surroundings before the start of the experiments.

This study will use a crude ethanolic extract from the floral parts of HRS at doses of 100 mg/kg, 250 mg/kg and 500 mg/kg to evaluate its antidepressant effects. The assessment will be conducted using three tests: the FST, the TST and the OFT. Fluoxetine will be purchased from a local medical shop and the usual dose of fluoxetine will be 15 mg/kg body weight in a diluted solution.

The instruments and equipment required for the experiments include a scalpel blade (size 11) for tail clipping, a disposable syringe (1 mL) for blood collection, a Soxhlet apparatus for plant extraction and a Buchi-type rotating vacuum evaporator for evaporating the extract.

Preparation of HRS: Two kilograms of HRS flowers will be washed and split into small pieces before being steeped in 100% ethanol for 15 days at room temperature, with occasional shaking.

Plant extraction: "Soxhlet extraction," or continuous hot percolation, will be employed in the extraction process. The HRS flowers will be pulverised and dried before being added to the Soxhlet apparatus.

The first non polar solvent used for the extraction will be ethanol, with the extraction taking place at 60°C. HRS Extracts (HRE) will be obtained and then evaporated at 40°C using a Buchi-type rotating vacuum evaporator. After that, the dried extract will be weighed and the following formula will be used to calculate the % yield for each extract [20,21]:

$$\%Yield=\frac{\text{Weight of extract}}{\text{The weight of plant material will be used}}\times 100$$

Acute oral toxicity test: The OECD 423 standards will be followed in conducting the acute oral toxicity investigation [22].

The procedure involves administering four dosage ranges: 5 mL/kg, 50 mg/kg, 300 mg/kg and 2000 mg/kg [Table/Fig-1] for toxicity assessments. The animals will fast overnight for approximately 18 hours. After the administration of the sample, each animal will be observed separately for 24 hours, with an additional four-hour observation postdosing to monitor for mortality during this period. In the limit test, Group IV male mice will receive a dose of 2000 mg/kg body weight. Samples will be withdrawn and sent for Liver Function Tests (LFT) and Complete Blood Count (CBC), while any behavioural changes will also be observed. Blood collection will be performed via the tail vein, with a sample volume of 1 mL. A local anaesthetic, a mixture of Lidocaine 2.5% and Prilocaine 2.5%, will be applied to the tail tip 30 minutes before sampling. Using a sterile scalpel blade (size 11), no more than 1 mm of tail tissue will be cut and the tail will be gently milked for blood collection. Haemostasis will be achieved by applying pressure using sterile gauze.

Investigations will include CBC and LFT. Following the administration of the test item, individual animals will be observed at 30 minutes, 1, 2, 3 and 4 hours postdosing on day 0 (the day of dosing) to 24 hours [Table/Fig-2]. Animals will also be observed for mortality on the day of dosing and for the next 24 hours. If there are any deaths reported among any of the groups, then we can proceed with the calculation of LD50 using probit value and graphical methods [23]. Videography will be conducted wherever necessary. Parameters for acute toxicity are described in [Table/Fig-2] [24].

Group	Sample sizes	Intervention	Dose and frequency	Follow-up	Animals to be sacrificed
I- Control group	10	Vehicle (distilled water)	ad libitum Orally stat (Aqueous Suspension)	Complete Blood Count (CBC) and Liver Function Test (LFT) on day 1 and day 3	NA
II- Experimental group	10	*Hibiscus rosa* Extraction-	50 mg/kg body wt. Orally stat (Aqueous Suspension)	CBC and LFT on day 1 and day 3	NA
III- Experimental group	10	*Hibiscus rosa* Extraction-	300 mg/kg body wt. Orally stat (Aqueous Suspension)	CBC and LFT on day 1 and day 3	NA
IV- Experimental group	10	*Hibiscus rosa* Extraction-	2000 mg/kg body wt. Orally stat (Aqueous Suspension)	CBC and LFT on day 1 and day 3	NA

[Table/Fig-1]: Mice will be divided into four groups as follows for acute toxicity.

S. No.	Parameters
1.	Incoordination of movements
2.	Salivation
3.	Lacrimation
4.	Piloerection
5.	Tremors
6.	Convulsions
7.	Paralysis
8.	Diarrhoea
9.	Feed intake
10.	Mortality

[Table/Fig-2]: Parameters for acute toxicity.

Behavioural assessment: Five groups will be formed from the selected rodents: control, standard (fluoxetine) and HRS (100 mg/kg, 250 mg/kg and 500 mg/kg), with each group containing six animals [Table/Fig-3].

Forced Induced Swimming (FST): Mice will be utilised to test the strategy. An open round and hollow holder with a width of 10 cm and a height of 25 cm, filled with 15 cm of water, will be used to house the mice at a temperature of 25±1°C. During the last four minutes of the six-minute testing session, the duration of observed inactivity will be recorded. If there are no aggressive or escape-directed activities (such as a mouse floating in the water without struggling), an observer will blindly score the immobile time [Table/Fig-4]. A decrease in the time the subject spends immobile during the FST will be used to gauge the efficacy of the treatment [25,26].

There will be two components to this test. The first portion is the pretest, which will be conducted the day before the experiment to select animals for follow-up research (Porsolt RD et al., 1977) [27]. The oral route will be utilised to administer all medications. After receiving their medication for an hour, rats will swim for six minutes in a glass tank that is 45 centimeters tall and 12 centimeters wide, with a water level that does not exceed 15 centimeters.

Tail Suspension Test (TST): The efficacy of the stimulant drug treatment in mice will be assessed through the TST as a testing protocol. For six minutes, mice will be suspended by their tails and behaviours related to escape will be evaluated. For this analysis, male mice will be divided into five groups of six mice each. Mice will receive the standard oral doses of fluoxetine (15 mg/kg) and HRS (100 mg/kg, 250 mg/kg and 500 mg/kg, respectively) [Table/Fig-5]. After an hour, mice will be hung by their tails 35 cm above the floor from the edge of a table for six minutes. The duration of their immobility will be measured for those mice that hang submissively [28]. Materials required will include a suspension box, tape, a timer, a video recording device, a white generator, cleaning supplies and climbing stoppers.

Group (6 mice per group)	Treatment	Dose	Animals to be sacrificed
I (Normal control)	Distilled water	(ad libitum) orally stat (Aqueous Suspension)	NA
II (Standard)	Fluoxetine	(15 mg/kg body wt.) orally stat (Aqueous Suspension)	NA
III (Experimental group)	*Hibiscus rosa sinensis* (HRS)	(100 mg/kg body wt.) orally stat (Aqueous Suspension)	NA
IV (Experimental group)	*Hibiscus rosa sinensis* (HRS)	(250 mg/kg body wt.) orally stat (Aqueous Suspension)	NA
V (Experimental group)	*Hibiscus rosa sinensis* (HRS)	(500 mg/kg body wt.) orally stat (Aqueous Suspension)	NA

[Table/Fig-3]: Mice will be divided into five groups as follows.

Treatment groups	Concentration (mg/kg)	Length of immobility Mean SEM (sec)	% Change
Control (6 mL/kg body wt.)			
Fluoxetine (15 mg/kg body wt.)			
Hibiscus rosa sinensis (HRS) L (100,250,500 mg/kg body wt.)			

[Table/Fig-4]: It will be tabulated in the following manner- {duration of immobility time in Forced Swim Test (FST)}.

Treatment groups	Concentration (mg/kg)	Length of immobility Mean SEM (sec)	% Change
Control (6 mL/kg body wt.)			
Fluoxetine (15 mg/kg body wt.)			
Hibiscus rosa sinensis (HRS) L (100,250,500 mg/kg body wt.)			

[Table/Fig-5]: Duration of immobility time in Tail Suspension Test (TST).

Open Field Test (OFT): In this trial, mice will be used to assess the impacts of an exploratory medication on their versatility. The open field equipment, made of white plywood, will measure 72 cm by 72 cm, with walls that are 36 centimeters high. During this investigation, mice will receive various treatments, including the standard drug fluoxetine (15 mg/kg) and the testing drugs, ethanolic extracts of HRS L. (100 mg/kg, 250 mg/kg and 500 mg/kg) [Table/Fig-6]. Next, the mice will be individually arranged in the middle of the large field and observed for five minutes before counting. Total Locomotion (TL), Peripheral Locomotion (PL) and Central Locomotion (CL) will be measured as the total number of squares crossed in both the external and internal planes. Other parameters that will be evaluated include defecation, grooming, learning and the number of times the animal rears up [29].

Treatments	Total Locomotion (TL)	Central Locomotion (CL)	Peripheral Locomotion (PL)	Learning (L)	Grooming (G)	Defeacation (D)
Control (6 mL/kg body wt.)						
Fluoxetine (15 mg/kg body wt.)						
Hibiscus rosa sinensis (HRS) L (100,250,500 mg/kg body wt.)						

[Table/Fig-6]: Duration of immobility time in Open Field Test (OFT).

Elevated plus-maze test: The elevated plus-maze is a primary tool for investigating the neuroprotective effects and anxiolytic responses of test drugs. It is used to assess nearly all variations of anxiolytic medications. In this test, rodents (mice and rats) face an approach-avoidance conflict when exposed to a novel maze, preferring enclosed arms over open ones due to their inclination for low, protected spaces. When encountering an open arm, rodents exhibit fear responses such as urination, freezing and immobility, along with increased plasma cortisol levels, indicating anxiety. The main advantages of this method are its quick and easy application without unpleasant stimuli (such as light or sound) and its reliability and consistency in studying anxiolytic actions and anxiety responses [30,31].

Grouping: Five groups of six Swiss albino mice each, weighing between 25 and 30 grams, will be randomly selected [Table/Fig-7].

Group (6 mice in each group)	Intervention	Dose and frequency	Duration (days)
I- Control group	Vehicle (distilled water)	6 mL/kg body wt. orally (Aqueous Suspension)	8
II- Experimental group	Fluoxetine	15 mg/kg body wt. Orally (Aqueous Suspension)	8
III- Experimental group	*Hibiscus rosa* Extraction-	100 mg/kg body wt. Orally (Aqueous Suspension)	8
IV- Experimental group	*Hibiscus rosa* Extraction-	250 mg/kg body wt. Orally (Aqueous) Suspension	8
V- Experimental group	*Hibiscus rosa* Extraction-	500 mg/kg body wt. orally (Aqueous Suspension)	8

[Table/Fig-7]: Grouping of *Hibiscus rosa* Extraction (HRE) for Elevated Plus Maze (EPM).

Procedure: It is suggested that the EPM test is an effective method for evaluating mice's learning and memory, which gauges transfer latency. Exteroceptive behavioural models, such as the EPM, assume that the body is not the source of stimuli. The raised plus-maze will consist of two open arms (16 cm×5 cm) and two enclosed arms (16 cm×5 cm×12 cm), giving the device the appearance of a plus sign.

The arms of the middle stage, which measure 5 cm by 5 cm, will extend, raising the labyrinth to a height of 25 cm above the floor. Each mouse will be positioned away from the central platform at the end of an open arm on the first day (the 7th day of medication therapy). The time it takes for the mouse to move all four of its legs into one of the covered arms is known as transfer latency. Each animal's transfer latency will be recorded on the first day [Table/Fig-8]. The subject's ability to retain the acquired task will be evaluated 24 hours after the first day of the trial, or on the eighth day of medication therapy. The mouse will return to its home cage after two more minutes of exploration.

STATISTICAL ANALYSIS

Statistics and Data (STATA) 10 software will be used for statistical analysis and data will be entered into a Microsoft Excel sheet. The paired t-test and ANOVA test will be employed to compare the antidepressant activity of HRS with Fluoxetine in order to assess toxicity and behavioural tests in mice. The parameters that will be compared include incoordination of movements, salivation, lacrimation, piloerection, tremors, convulsions, paralysis, diarrhoea, feed intake and mortality. A p-value ≤0.05 will be considered significant.

Treatments	Total Locomotion (TL)	Central Locomotion (CL)	Peripheral Locomotion (PL)	Learning (L)	Grooming (G)	Defeacation (D)
Control (6 mL/kg body wt.)						
Fluoxetine (15 mg/kg body wt.)						
Hibiscus rosa sinensis (HRS) (100, 250, 500 mg/kg body wt.)						

[Table/Fig-8]: Duration of immobility time in Elevated Plus Maze (EPM) test.

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