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## COMBINED EFFECT OF JUSTICIA ADHATODA AND CROCUS SATIVES ON ZEBRAFISH MODEL FOR CONITIVE ENHANCEMENT

V Ragini\*, Dhanish Kumar, K Vandana, Kumar Nandini, Mangapuram Kavyasree, Munagala Rajavardhan  
Department of Pharmacology, Sri Venkateswara College of Pharmacy, Chittoor, Andhra Pradesh, India.

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**\*Corresponding author**

Dr. V Ragini

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### Abstract

Brain-related problems can include brain injuries, brain tumors, and brain diseases. They can also include mental health disorders such as Depression, Anxiety, Bipolar disorder, Post- traumatic stress disorder (PTSD). This study explores the combined cognitive-enhancing potential of Justicia adhatoda and Crocus sativus using a zebrafish (*Danio rerio*) model. Both plants are known for their neuroprotective and mood-stabilizing properties. Methanolic extracts were prepared and tested through behavioral assays, including the Open Field Test (OFT) and Novel Object Recognition Test (NORT), which assess anxiety, locomotor activity, and memory function. Results indicated that the combination group exhibited significantly higher central zone activity and recognition index compared to the control and individual treatment groups. Justicia adhatoda enhanced cognitive performance through cholinergic modulation and antioxidant effects, while Crocus sativus acted via serotonergic pathways and BDNF upregulation. Their synergistic interaction led to improved memory, reduced anxiety-like behavior, and better cognitive processing. This study highlights the therapeutic potential of combining these plant extracts for CNS enhancement and supports their use as a natural, multi-targeted alternative for managing cognitive disorders. Further research is recommended to validate these findings in clinical settings.

**Keywords:** Justicia adhatoda, Crocus sativus, Open field Test, Novel Recognition Test, Zebra fish model, Neuroprotection.

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### Introduction

Brain-related problems can include brain injuries, brain tumors, and brain diseases. They can also include mental health disorders such as Depression, Anxiety, Bipolar disorder, Post- traumatic stress disorder (PTSD) [1]. Depression is a mood disorder characterized by persistently low mood and a feeling of sadness and loss of interest. More than 264 million people suffer from depression worldwide. Causes of depression such as Hormones, stress, and trauma, substance use, physical health condition and also seasonal changes. It aimed at improving cognitive abilities like memory, attention and reasoning [2].

The central nervous system (CNS) is responsible for regulating essential cognitive functions such as learning, memory, and behavior. Cognitive decline caused by aging, neurodegenerative diseases, or stress has led to increased interest in natural compounds with cognitive-enhancing potential. Justicia adhatoda, rich in vasicine, and Crocus

sativus (saffron), containing active constituents like crocin and safranal, are known for their neuroprotective, antioxidant, and memory-enhancing properties [3-5]. This study explores the combined effect of these two medicinal plants on cognitive enhancement using a zebra fish (*Danio rerio*) model, which is widely used in neurobehavioral research due to its genetic similarity to humans. Behavioral tests such as the novel tank recognition test and open field test assays are employed to assess the synergistic effects on cognitive function.

### Materials & Methodology

#### Plant Selection

Plant selection is based on several advantages of the species. Justicia adhatoda species reported with various therapeutic activities like anti-inflammatory activity, antimicrobial activity, antiviral activity etc from the literature review & Crocus sativus species have the

various therapeutic activities such as antioxidant and antidepressant effects.

#### Plant Collection

*Crocus sativus* is obtained from Local market, Chittoor and *Justicia adhatoda* is obtained from ecommerce platform. The zebra fish [*Dania rerio*] species is reported with various therapeutic activities like anti depressant to study anxiety and stress responses. It is collected from Local Fish market, Tirupati. The Open Field Test and Object Recognition Test on Zebra Fish were performed.

#### Plant authentication

The plant material is authenticated by Dr.K.Madhava Chetty, Assistant professor, Department of Botany, Sri Venkateswara University, Tirupati.

#### Plant Extraction

##### Extraction of *Crocus sativus*

Two grams of dried stigmas were suspended in a 40 ml mixture of methanol and water (50:50) and stirred for 24hr at 4°C in a dark room. The solution was filtered and the methanol was evaporated at 40°C using rota vapour. The obtained solution was subjected to refrigeration before lyophilization. Mass yield of saffron extract was 8%w/w.

##### Extraction of *Justicia adhatoda*

The ground powder (20g) of *J. adhatoda* leaves was taken and mixed with 200ml of methanol in a beaker at room temperature and kept in a shaking incubator adjusted at 150rpm speed for 24hrs. After 24hrs the extract was collected and filtered using whatmann no1 filter paper and was vacuumed and dried using a rotary evaporator more over. The obtained extract was stored 4°C for further use.

Table 1. Experiment Groups

S.No	GROUP	TREATMENT
1	Control	No treatment or vehicle
2	<i>Justicia adhatoda</i>	<i>Justicia adhatoda</i> extract
3	<i>Crocus sativus</i>	<i>Crocus sativus</i> extract
4	Combination	<i>Justicia adhatoda</i> extract + <i>Crocus sativus</i> extract

#### Evaluation of Cognitive Enhancement Potential

##### 1. Object Recognition Test

Zebra fish are acclimated to a testing tank without objects, and then exposed to two identical objects. After exploration, one object is replaced with a novel one following a retention interval. Time spent exploring each is recorded to assess memory [6].

##### ➤ Locomotor activity

Increased Time near the Novel Object Suggests improved cognitive function and memory. It indicates a successful recognition of a previously encountered object. No Preference for the Novel Object Suggests memory impairment or cognitive deficits. It is common in

neurodegenerative disease models or drug-induced cognitive decline [7].

##### ➤ Statistical Analysis

Data are analyzed using Graph Pad Prism with appropriate utilization by using Parametric Paired t-test (if same fish before vs. after), independent t-test (if comparing two groups), one-way ANOVA for multiple groups). Non-Parametric tests (Wilcoxon signed-rank test (for paired data) or Mann-Whitney U test (for independent samples)).

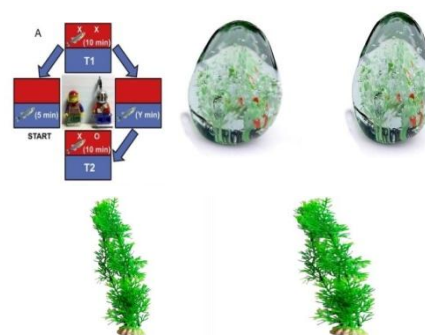


Figure 1. Object Recognition Test

##### 2. Open field test

Zebrafish were tested for boldness in a black-covered open field arena. The open-field test arena consisted of a clear, plastic tank (30cm x 30cm x 10cm) with water depth 8 cm covered on all sides with black plastic. Three replicate arenas were constructed to allow testing of multiple fish at the same time. The arenas were placed on the floor and were positioned in such a way that no direct light fell on the arenas and no shadows were created by the arena walls. After 2 minutes in a center cylinder, fish were released and behavior recorded for 5 minutes. Trials were filmed from above, and fish were transferred to holding tanks [8].

##### ➤ Locomotor activity

Increased Activity (Higher Exploration) suggests reduced anxiety or hyperactive behavior. It can be observed with stimulants, anxiolytics, or genetic modifications affecting activity. Decreased activity (Lower Exploration) indicates increased anxiety or sedation. It is common with stress, anxiogenic drugs, or neurological impairments - Thigmotaxis (Preference for the Edges) [9, 10].

##### Statistical Analysis

Data were analyzed using GraphPad Prism with appropriate statistical tests & utilization by using Parametric Paired t-test (if same fish before vs. after), independent t-test (if comparing two groups), one-way ANOVA for multiple groups) Non-Parametric tests (Wilcoxon signed-rank test (for paired data) or Mann-Whitney U test (for independent samples)).

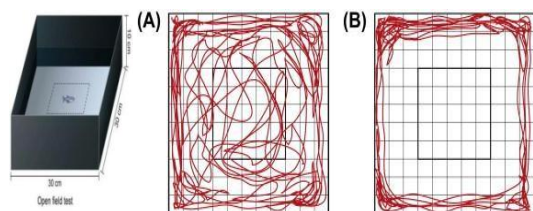


Figure 2. Open field test

In the open field test (OFT) and novel object recognition test (NORT) using zebra fish, when *Justicia adhatoda*, *Crocus sativus*, and their combination are administered in control, test, and combination groups, the expected behavioral outcomes related to cognitive enhancement and antidepressant activity are as follows:

#### 1. Control Group (No treatment or vehicle only):

**Purpose:** Serves as the base line comparison.

**Treatment:** No drug treatment

**Effect:** May exhibit thigmotaxis (preference for edges) indicating natural stress response.

Limited cognitive activity observed.

Expected Behavior: Normal locomotion and behavior.

Mild anxiety and typical cognitive performance in behavioral tests like OFT or NORT.

#### 2. Test Group1: *Justicia adhatoda*

**Treatment:** Administered an extract of *Justicia adhatoda* (specific dose based on Effective concentration).

#### Expected Behavioral Changes

Increased locomotion and exploratory behavior.

Reduced anxiety-like behavior (more time in center zone)

#### Expected Outcome

Moderate improvement in cognitive function via AChE inhibition.

Slight reduction in anxiety and enhanced memory in behavioral assays.

#### 3. Test Group 2: *Crocus sativus*

**Treatment:** Administered extract of *Crocus sativus* (crocin/safranal-rich fraction).

#### Expected Behavioural Changes

Greater movement and swim patterns suggesting antidepressant-like effects.

Reduced freezing episodes (a marker of anxiety/depression in zebrafish).

#### Expected Outcome

Significant improvement in memory and mood. Better performance in NOR (novel object recognition) and OFT (more exploratory behavior).

#### 4. Combination Group: *Justicia adhatoda*+*Crocus sativus*

**Treatment:** Co-administration of both *Justicia adhatoda* and *Crocus sativus* extracts (either half doses or full synergistic doses).

#### Expected Behavioral Synergy

Increased time in the center zone

Higher total distance moved.

#### Expected Outcome

Greater reduction in anxiety and depressive-like behavior. Enhanced cholinergic, serotonergic, and antioxidant effects.

## Results & Discussion

The combination of *Justicia adhatoda* and *Crocus sativus* demonstrated a significant enhancement in cognitive performance in the zebra fish model when evaluated using the Open Field Test (OFT) and Novel Object Recognition Test (NORT). Both herbs improve brain function in zebra fish models, but *Crocus sativus* (saffron) demonstrated superior efficacy.

Table 2. Cognitive Performance Metrics in Zebra Fish

GROUP	OPEN FIELD TEST (TIME IN CENTRAL ZONE, SECONDS) MEAN±SD	NOVAL OBJECT RECOGNITION TEST (RECOGNITION INDEX %) MEAN±SD
Control	14 ±1.6	48±3.5
<i>Justicia adhatoda</i>	21.8±1.1	58.7±2.8
<i>Crocus sativus</i>	25±1.1	65.1±3.1
Combination	32.4±1.1	78.9±2.6

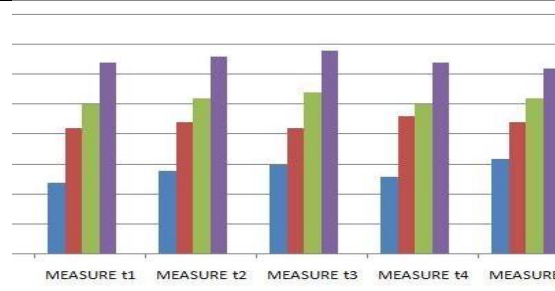


Figure 3. Cognitive enhancement activity

*Justicia adhatoda* alone shows mild cognitive benefits. *Crocus sativus* alone provides moderate improvements in memory and behavior. The combination exhibits superior effects, indicating a synergistic interaction that enhances learning, memory, and reduces anxiety in zebra fish model.

The research primarily explores how *Justicia adhatoda* and *Crocus sativus* affect cognitive performance, memory, anxiety-like behavior, and locomotion in zebrafish using two behavioral tests:

Open Field Test (OFT) – to assess anxiety, locomotor activity, and exploration. Novel Object Recognition Test (NOR) – to evaluate memory, learning, and discrimination abilities.

## Behavioral and Cognitive Effects

Zebrafish reared with *Crocus sativus* and *Justicia adhatoda* showed better memory and object discrimination abilities compared to control fish. Fish given enriched environments with these herbs were more active, explored more, and showed less fear in open spaces (indicating lower anxiety levels). *Crocus sativus* had a stronger impact than *Justicia adhatoda* in reducing anxiety and improving cognitive functions.

### Open Field Test (OFT)-Anxiety and Activity

Interpretation: Crocus Sativus made fish more confident and active. Justicia adhatoda also helped, but less effectively.

### Novel Object Recognition (NOR) - Memory and Learning

Interpretation: Crocus Sativus shows great enhancement in memory, learning and neurochemical support than Justicia adhatoda

### Combination Group (*Justicia adhatoda* + *Crocus sativus*)

Synergistic cognitive enhancement, Greater reduction in anxiety and depressive-like behavior, Better learning and memory performance than either herb alone, Enhanced cholinergic, serotonergic, and antioxidant effects., Likely synergistic effects from:, Justicia's cholinergic and antioxidant effects, Crocus's serotonergic and neurotrophic effects.

In this study, we used zebrafish to explore the effect of two herbal extracts—*Justicia adhatoda* and *Crocus sativus*—might help improve memory and reduce anxiety. Zebrafish are useful in brain studies because their behavior reflects brain function. At the start, untreated zebrafish were less active, showed little curiosity, and had poor memory, especially in the Open field Test (OFT) & Novel Object Recognition Test (NORT), where they showed limited interest in new objects.

When the fish were treated with *Justicia adhatoda*, they showed some improvement in memory. This happened because the extract helped block an enzyme called acetylcholinesterase, which usually breaks down acetylcholine—a chemical in the brain important for learning and memory. So, more acetylcholine stayed active, which helped the fish remember better. The fish also seemed less anxious and had lower levels of inflammation and stress in the brain.

On the other hand, the fish treated with *Crocus sativus* (commonly known as saffron) showed even stronger effects. Their memory and learning improved a lot, and their brains had more serotonin, dopamine, and BDNF (a protein that helps brain cells grow and stay healthy). These fish were less anxious and less depressed. They also explored their surroundings more and showed a strong preference for new objects in the NOR test.

The most impressive results came when both extracts were used together. The combined treatment worked better than using either extract alone. It not only improved memory and reduced anxiety but also helped brain chemicals and protective pathways work together. The fish showed better behavior in all the tests—they explored more, had less fear, and remember new objects better. So, when *Justicia adhatoda* and *Crocus sativus* are used together, they support each other's effects. One helps increase acetylcholine (important for memory), while the other boosts brain chemicals like serotonin and BDNF (which help reduce anxiety and support brain health).

Together, they create a powerful effect that improves memory, reduces stress, and protects the brain.

### Conclusion

The study shows that using both *Justicia adhatoda* and *Crocus sativus* together could be a promising natural treatment to help with brain function and mental health issues. This combination can improve memory, learning, and emotional balance, and also protect brain cells. It works in many ways—by increasing important brain chemicals, reducing stress and anxiety, and supporting brain health through natural healing paths.

These herbs might work even better than some current medicines used for brain-related conditions, and they could be safer too. Because they act on several systems in the brain at once (like memory, mood, and protection), they have the potential to be a new kind of brain-boosting and brain-healing treatment. This makes them strong candidates for future medicine to treat problems like memory loss, anxiety, and other disorders related to the brain.

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### Inform Consent and Ethical Considerations

Not Applicable

### Conflict of Interest

The authors declare no conflict of interest.

### Author Contribution

Concept: Dr. V Ragini, design: Dr. V Ragini, data collection: Dhanish Kumar, K Vandana, Kumar Nandini, Mangapuram Kavyasree, Munagala Rajavardhan, analysis: Dr. V Ragini, writing: Dhanish Kumar, K Vandana, Kumar Nandini, Mangapuram Kavyasree, Munagala Rajavardhan.

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