Standardized Extract from the Gotu Kola Leaves Improves Suicidal Behavior in Stressed Rats Subjected to Social Isolation

Prasad Arvind Thakurdesai1*, Savita Raju Nimse1, Padmaja Santosh Kore2 and Urmila Manoj Aswar3

1Department of Scientific Affairs, Indus Biotech Limited, Pune, India.
2Department of Pharmacology, Modern College of Pharmacy, Nigdi, Pune, India.
3Department of Pharmacology, Poona College of Pharmacy, Bharati Vidyapeeth Deemed University, Pune, India.
*Corresponding Author E-mail: prasad@indusbiotech.com

https://dx.doi.org/10.13005/bpj/2896
(Received: 12 December 2023; accepted: 09 May 2024)

The leaves of *Centella asiatica* L. Urban (*C. asiatica*) and their bioactive compounds, triterpenoids asiaticoside (AS) and madecassoside (MA), are effective in reducing psychological stress and associated behavioral disorders in the conducted in vivo research. The present study evaluated AS+MA-based standardized *C. asiatica* leaves extract (INDCA) on social isolation stress (SIS)-induced suicidal behavior-related traits in laboratory rats. Male rats (*n*=6) were randomized, grouped, and individually caged for seven days for stress induction. For the next seven days (D7 to D14), rats were orally administered vehicle (stress control), positive control (fluoxetine, 30 mg/kg), and or INDCA (3, 10, 30 mg/kg) once a day. A separate group of rats without isolation stress (normal rats) was maintained. The scores for suicidal behavior-related traits, such as aggression, impulsivity, irritability, learned helplessness, and plasma cortisol, were measured after 14-day treatment. The stress control group showed a significant increase in aggression, irritability (total score), learned helplessness (escape latency, escape failure, and recovery attempts), and plasma cortisol levels, which confirmed suicidal behavior-related traits. The INDCA-treated rats showed a dose-dependent reduction in stress-induced behavioral traits and elevated plasma cortisol levels. In conclusion, subacute administration of INDCA showed amelioration of suicidal behavior in SIS in laboratory rats and suggested a promising natural and safe option for the management of stress-induced behavioral disorders, including suicidal behavior.

Keywords: Cortisol; Gotu kola leaves; Social Isolation; Stress Disorders; Suicidal Ideation.
limitations in children and young adults due to suicidal ideation. Many psychological disorders are stress-related and mediated through hypothalamic–pituitary–adrenal (HPA) axis. The brain serotonergic system, which is responsible for stress-related functions, is strongly correlated with mood and anxiety. Therefore, antidepressant agents with stress-relieving properties but without the side effects of suicidal traits are required.

Social isolation is a factor in suicidal behaviors. In addition, social connections in the community may protect stressed individuals from developing suicidal desires. Consistent with the “stress reactivity” concept, cortisol has been reported as a dependable indicator of HPA axis function in MDD. Therefore, stress and cortisol reduction are essential targets for the management of many psychological disorders, including suicidal ideation.

Plant-derived natural products with evidence of brain tonic and stress-relieving properties have been explored to manage psychological disorders. Gotu kola, scientifically known as Centella asiatica L. Urban (CA), is a promising plant source to alleviate stress-induced behavioral traits such as anxiety, depression and mood in healthy volunteers, and mild cognitive decline in elderly individuals. C. asiatica is a major medicinal herb used to alleviate anxiety symptoms in traditional medicine, such as Ayurveda and Chinese medicines. Previous studies have demonstrated the neuroprotective properties of standardized C. asiatica extracts against neurotoxin-induced oxidative stress and mitochondrial dysfunction in the brain, which may be responsible for anti-aging potential.

Triterpenoids, such as asiaticoside (AS) and madecassoside (MA), are major bioactive components of C. asiatica leaves with stress and anxiety-reducing and anti-aging properties. A triterpenoid-based extract from C. asiatica leaves (INDCA) has shown antidepressant effects in rats with chronic depression induced by olfactory bulbectomy. The beneficial effects of C. asiatica triterpenoids are reportedly achieved by improving the function of the HPA axis and enhancing the levels of monoamine neurotransmitters.

In rodents, social isolation induces various behavioral changes, including anxiety, impulsivity, aggression, and suicidal traits. Socially isolated rats are valuable animal models for exploring options against stress-induced behavioral traits, including suicidal ideation. Existing clinical and preclinical scientific evidence indicates the potential of INDCA as an antidepressant and anxiolytic agent with stress-relieving properties. However, a detailed exploration of INDCA to manage suicidal behavior has not yet been performed. Therefore, the present study aimed at pharmacological assessment of oral INDCA supplementation on aggression, impulsivity, irritability, and learned helplessness induced by social-isolation stress (SIS) in laboratory rats.

**MATERIALS AND METHODS**

**Chemicals**
Fluoxetine and cortisol kits (EIA-1887) were obtained from Cadila Pharma (Ahmedabad, India) and DRG Diagnostics (Marburg, Germany), respectively. Indus Biotech Limited (Pune, India) provided the test compound, INDCA, a standardized C. asiatica leaves extract, with a total triterpenoid content of 75.5% (35.88% MA and 39.62% AS) by high-performance liquid chromatography as previously reported. Fresh solutions of fluoxetine and INDCA were prepared daily immediately before oral administration to the rats.

**Animals**
Sprague-Dawley rats (male, weighing 200–250 g of 2-3 months of age) were procured and maintained in polypropylene cages in rooms with ambient temperature, humidity, and light: dark cycle following ethical standards and regulations for animal experiments in India. All animal experiments were compiled the norms of “Committee for the Purpose of Control and Supervision of Experimental Animals” (CPCSEA) with protocol approval (No: SIOP/IAEC/2012/70) by the “Institutional Animal Ethics Committee” of Sinhgad Institute of Pharmacy (Narhe, Pune, India).

**Induction of SIS and treatments**
Rats were acclimatized to the testing room by moving them from the animal house an hour before testing. The rats were divided into six groups of six rats each. To prevent the influence of diurnal rhythms, experiments were conducted at 8:00 AM and 1:00 PM. To avoid personal bias,
a person unaware of the treatments recorded the observations.

One group of rats, G1, called the normal, did not undergo SIS42 and received vehicle (1 ml/kg). The remaining groups of rats (G2–G6) were housed individually throughout the experiment received SIS, and 14-days of oral treatment as follows:

- G2: Stress control - Received vehicle at dose 1 ml/kg;
- G3: Fluoxetine (20) - Received positive control, Fluoxetine at dose 20 mg/kg;
- G4: INDCA (3) - Received INDCA at dose 3 mg/kg;
- G5: INDCA (10) - Received INDCA at dose 10 mg/kg;
- G6: INDCA (30) - Received INDCA at dose 30 mg/kg.

Aggression and impulsivity in SIS-induced rats

The resident-intruder paradigm measures aggression-related parameters43,44. First, one rat was confined to the cage for 5 min, after which the intruder rats were brought into the same cage. A video tracking apparatus (ASTMD2467-SCH80, VJ Instruments, Akola, India) was used to record aggressive behavior for 10 min. Aggressive behavior (attack bites, tail rattling, wrestling, and chasing) was noted on days 1 (D1), Day-7 (D7), and Day-14 (D14) of isolation. The sum of all scores was considered the total aggression score. The time the intruder mouse took to initiate an attack (attack latency) was documented and used to indicate impulsiveness42.

Irritability in SIS-induced rats

The irritability of individual rats was recorded on D1, D7, and D14 immediately after the measurement of aggression scores using a reported method45. Each rat received an individual puff of uncomfortable air blown through a straw on its neck. The enhanced reaction to stimuli by rats is noted as “irritable” behavior and scored as irritability score (intensity of the response) using a six-category scale (0 to 6)45. Adding individual irritability scores represented that rat’s “total irritability score”.

Learned helplessness in SIS-induced rats

The effects on stress-induced learned helplessness were assessed immediately after irritability measurements on D14 of the experiment. The investigations were performed active avoidance paradigm46 using an active avoidance chamber (Model-DS163, INCO, Ambala India) with 15 attempts (33 s) for each rat. During the first 3 seconds, no shock was applied. In the absence of escape, an electric current (0.8 mA for 15 s) was administered at an interval of 45 s between each stimulus. In the event of the jump, the process was terminated and recorded as an “escape,” and the latency was recorded. Failure to jump the pole (helplessness) occurred, and the attempt was registered as an “escape failure,” and the number was recorded. Attempts to recover (i.e., when the rat learned to escape and showed escape instead of escape failure) were recorded individually.

Plasma cortisol levels in SIS-induced rats

Cortisol levels were assessed on D14 in plasma isolated from blood samples of rats (collected under anesthesia) using an ELISA kit (DRG Diagnostics, Germany), following the instructions of the manufacturer and supplier.

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). The total scores for aggression, impulsivity, and irritability were analyzed using two-way repeated-measures analysis of variance (ANOVA) and Bonferroni multiple comparisons. Kruskal-Wallis ANOVA and Dunn’s multiple comparison tests were used to analyze data obtained on D14 from the learned helplessness test (escape latency, failure numbers, and number of recovery attempts). Data of plasma cortisol measurements were analyzed using one-way ANOVA followed by Dunnett’s test. Prism (v. 6.0, GraphPad Inc., San Diego, USA) was used for data analysis. Differences were considered statistically significant at P < 0.05.

RESULTS

Effects on total aggression scores

The data analysis of the total aggression scores during the resident-intruder paradigm is organized in Table 1. No significant change was observed between any treatment groups on D1 and D7. On D14, stress control showed a significant (P < 0.001) increase in aggression scores compared to normal rats, whereas fluoxetine and INDCA (all tested doses) treated groups showed a decline in total aggression scores (vs. stress control).
Table 1. Effect on total aggression scores and impulsivity (attack latency) in social-isolation stress induced rats

<table>
<thead>
<tr>
<th>Treatment (Dose in mg/kg, p.o.)</th>
<th>Parameter score (Mean ± SEM)</th>
<th>Total Aggression score</th>
<th>Impulsivity - Attack Latency (Sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D1</td>
<td>D7</td>
<td>D14</td>
</tr>
<tr>
<td>Normal</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Stress control</td>
<td>2.33 ± 1.67</td>
<td>12.33 ± 2.76</td>
<td>49.17 ± 6.82***</td>
</tr>
<tr>
<td>Fluoxetine (30) + Stress</td>
<td>5.33 ± 2.72</td>
<td>18.00 ± 1.75</td>
<td>30.83 ± 7.30**</td>
</tr>
<tr>
<td>INDCA (3) + Stress</td>
<td>5.33 ± 2.78</td>
<td>12.33 ± 2.38</td>
<td>19.83 ± 1.35***</td>
</tr>
<tr>
<td>INDCA (10) + Stress</td>
<td>13.50 ± 5.86</td>
<td>23.50 ± 2.60</td>
<td>13.17 ± 2.69***</td>
</tr>
<tr>
<td>INDCA (30) + Stress</td>
<td>12.67 ± 4.33</td>
<td>17.00 ± 2.97</td>
<td>16.00 ± 3.73***</td>
</tr>
</tbody>
</table>

n=6, Total aggression score is calculated as the sum of scores from attack bites, tail rattling, wrestling and chasing behavior. *P < 0.05, **P < 0.01, ***P < 0.001 (vs. Normal), #P < 0.05, ###P < 0.001 (vs. stress control) at respective day (two-way repeated measures ANOVA and Bonferroni multiple comparisons test)
significant (P < 0.05, P < 0.001) reduction in the number of attempts to recover was observed in the INDCA (10) and INDCA (30) groups but not in the INDCA (3) group.

**Effects on plasma cortisol levels**

The data obtained from plasma cortisol measurements are shown in Fig. 1. The mean plasma cortisol concentration in the normal group rats was 14.76 ng/ml. In the stress control rats, mean plasma cortisol concentration significantly (P < 0.001) increased to 176.3 ng/ml (vs. Normal). Fluoxetine or INDCA treatment group showed the mean plasma cortisol levels to 271.6 ng/ml, 82.49 ng/ml, 69.14 ng/ml, and 14.35 ng/ml, respectively, which are significantly (P < 0.001 vs. stress control).

**Table 2. Effect of on Irritability score in social-isolation stress induced rats**

<table>
<thead>
<tr>
<th>Treatment (Dose in mg/kg, p.o.)</th>
<th>D1 (Mean ± SEM)</th>
<th>D7 (Mean ± SEM)</th>
<th>D14 (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.33 ± 0.49</td>
<td>1.00 ± 0.37</td>
<td>0.67 ± 0.33</td>
</tr>
<tr>
<td>Stress control</td>
<td>0.83 ± 0.40</td>
<td>3.67 ± 0.49</td>
<td>5.17 ± 0.54</td>
</tr>
<tr>
<td>Fluoxetine (30) + Stress</td>
<td>1.17 ± 0.54</td>
<td>3.50 ± 1.20</td>
<td>5.00 ± 0.63</td>
</tr>
<tr>
<td>INDCA (3) + Stress</td>
<td>0.50 ± 0.22</td>
<td>1.17 ± 0.31</td>
<td>2.50 ± 1.15</td>
</tr>
<tr>
<td>INDCA (10) + Stress</td>
<td>1.50 ± 0.81</td>
<td>2.33 ± 1.12</td>
<td>1.50 ± 0.50</td>
</tr>
<tr>
<td>INDCA (30) + Stress</td>
<td>1.33 ± 0.84</td>
<td>0.67 ± 0.67**</td>
<td>0.33 ± 0.21***</td>
</tr>
</tbody>
</table>

n=6, Total Irritability score is calculated as the sum of scores obtained from startle, snout, biting, and resistance and vocalization responses. # P < 0.05, ** P < 0.001 vs. Normal, * P < 0.05, ** P < 0.01, *** P < 0.001 (vs. stress control) on the respective day (Two-way repeated measures ANOVA and Bonferroni multiple comparisons test).

**Table 3. Effects on learned helplessness during active avoidance test in social-isolation stress induced rats**

<table>
<thead>
<tr>
<th>Treatment (Dose in mg/kg, p.o.)</th>
<th>Parameter Value on D14 (Mean ± SEM)</th>
<th>Escape latency (sec)</th>
<th>Failure attempts (number)</th>
<th>Attempts to recover (number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td>64.20 ±7.83</td>
<td>0.00 ±0.00</td>
<td>5.80 ±0.20</td>
</tr>
<tr>
<td>Stress control</td>
<td></td>
<td>183.20 ±9.59**</td>
<td>5.60 ±0.24**</td>
<td>16.00 ±0.00**</td>
</tr>
<tr>
<td>Fluoxetine (30) + Stress</td>
<td></td>
<td>174.00 ±6.04</td>
<td>2.00 ±0.37</td>
<td>13.00 ±0.33</td>
</tr>
<tr>
<td>INDCA (3) + Stress</td>
<td></td>
<td>178.70 ±18.96</td>
<td>1.67 ±0.21</td>
<td>12.50 ±0.56</td>
</tr>
<tr>
<td>INDCA (10) + Stress</td>
<td></td>
<td>102.50 ±19.33**</td>
<td>1.50 ±0.22</td>
<td>9.33 ±0.88**</td>
</tr>
<tr>
<td>INDCA (30) + Stress</td>
<td></td>
<td>65.17 ±10.24***</td>
<td>0.00 ±0.00***</td>
<td>7.00 ±0.68***</td>
</tr>
</tbody>
</table>

n=6, The score in 15 attempts for each trait is recorded. * P < 0.05, ** P < 0.01, *** P < 0.001 (vs. Normal), # P < 0.5, ** P < 0.01, *** P < 0.001 (vs. stress control) of respective parameters (Kruskal-Wallis ANOVA and Dunn’s multiple comparison test).

**DISCUSSION**

As suicide is a complex behavior, specific animal models have not been developed. However, four suicidal behavioral traits, namely aggression, irritability, hopelessness, helplessness, and impulsivity, are known to be closely related to suicidal ideation. Simultaneously, social isolation in rodents induces the same behavioral changes as suicidal ideation, including impulsivity and aggression and mood-related behavior. Social isolation is a common cause of anxiety disorders in clinical settings. Due to its non-physical nature, social isolation can alleviate pain and stimulate stress responses in animals by activating the HPA axis. Antidepressants, particularly SSRIs, induce...
stress-related behaviors in animal models\textsuperscript{31}. The current study utilized social isolation as a stressor because of its association with clinical settings\textsuperscript{42}. The most promising endpoints related to suicidal behavior in animals are cortisol levels, social stress response, and aggression/impulsivity traits involving the serotonergic system \textsuperscript{52}. Therefore, we measured the effects of oral supplementation of INDCA on aggression, impulsivity, irritability, helplessness, and plasma cortisol levels in stress-induced rats as indicators of suicidal ideation. The test compound, INDCA, was extract powder prepared from authenticated and dried leaves of Centella asiatica by hydroalcoholic extraction and purification with column chromatography based on earlier reported procedure\textsuperscript{35}.

Almost all animal species exhibit aggressive behavior as a natural trait \textsuperscript{53}. The resident-intruder paradigm, considered one of the most reliable methods for assessing aggressive behavior-related traits, was employed in the current study\textsuperscript{44, 53}. Our findings, which indicate higher total aggression scores in stress control, are consistent with a previous study\textsuperscript{42}. In addition, INDCA treatment prevented stress-induced increases in total aggression scores.

The serotonergic system in the brain influences aggression by regulating the release and firing of serotonergic neurons through presynaptic 5-HT\textsubscript{1A} auto receptors\textsuperscript{54}. Specific 5-HT\textsubscript{1A}/1B receptor agonists potently reduce aggressive behavior without causing motor retardation or sedative effects\textsuperscript{55}. Furthermore, fear, anxiety, and the consequent defensive behavior in aggression are regulated by 5-HT\textsubscript{1A} receptor expression in the brain \textsuperscript{56}. The 5HT\textsubscript{1A} receptor agonist property was proposed as a possible mechanism of action of C. asiatica extract as an antidepressant\textsuperscript{57} and an anti-migraine\textsuperscript{58} agent. The results of the present study provide additional support for the 5HT\textsubscript{1A}/1B receptor-mediated mechanism of INDCA against other stress-related conditions, especially aggression.

Impulsivity is an essential trait in the development of several neuropsychiatric conditions, including suicidal ideation\textsuperscript{59}. The tendency towards impulsive behavior is often observed alongside other mental health conditions such as mood disorders and suicidal behavior\textsuperscript{59}. Decreased attack latency (latency for first attack from intruder mouse entry) indicates the impulsivity trait of aggression\textsuperscript{42}. A substantial increase in impulsivity on D14 in stress control was noted in the present study. However, Fluoxetine or INDCA failed to demonstrate any impact on impulsivity, probably because of the brief duration of the treatments and the presence of stress as reported\textsuperscript{60}.

![Fig. 1. Effect on plasma cortisol levels in social-isolation stress-induced rats. n = 6, Data as mean ± standard error of the mean (SEM) and was analyzed by one-way ANOVA followed by Dunnett’s test. $$$ significant increase (P < 0.001) vs. Normal, $$ significant increase (P < 0.001) vs. Stress control, *** significant decrease (P < 0.001) vs. Stress control, Numbers in parentheses indicate oral dose in mg/kg. FLU- Fluoxetine, SIS – Stress-induced isolation](image-url)
Irritability is a state of mind characterized by poor temper control and irreversible verbal or behavioral outbursts. The ability to control one’s emotional responses is often disregarded and frequently observed in individuals who display impulsive behavior. Several studies have demonstrated a strong correlation between irritability, suicidal ideation, and suicide attempts. Irritability measurement in rodents uses an animal’s reaction to mild touch or sound. This is complicated by the subjective nature of mental states. The current study employed a 6-point scale to assess overall irritability in rats following exposure to unpleasant stimuli. In the present study, SIS increased irritability in the stress control group, whereas INDCA treatment prevented stress-induced irritability in a dose-dependent manner. The absence of a substantial impact of fluoxetine on SIS-induced irritability is consistent with previous research.

Hopelessness, or helplessness, is one of the most notable features of suicidal ideation. Helplessness is defined as deep pessimism about the future. Learned helplessness is the most validated measure and is often used as a representative trait for hopelessness assessment. The active avoidance paradigm, which uses animals' natural aversion to foot shocks, has been used to investigate learned helplessness. The animal will eventually display a “despair-like” behavior when the stimulus is inescapable and can no longer attempt to escape. The 14-days SIS significantly enhanced escape latencies and failure attempts in stress-control rats, confirming the induction of learned helplessness. A significant prevention of learned helplessness in rats following oral treatment with INDCA (10 or 30 mg/kg) was observed.

Exposure to prolonged social stress and elevated corticosteroid levels can impair stress adaptation mechanisms and contribute to learned helplessness. Furthermore, 5-HT1A activation in the dorsal hippocampus leads to enhanced stress adaptation. The behavioral deficits of SIS resulted in escape attempts from restrained space, which are reversed by 5HT1A agonist. INDCA prevented learned helplessness-induced disturbances in the stress adaptation mechanism, perhaps by reducing cortisol and 5HT1A receptor activation.

SIS is recognized for its ability to cause hypothalamic-pituitary-adrenal (HPA) axis hyperresponsiveness and contributes to hippocampal neuronal degeneration. Many studies have emphasized the significant role of the hippocampus under stressful conditions. The hippocampus controls adrenocorticotropic hormone (ACTH) and cortisol secretion via the HPA axis.

Stress responses were reliably correlated with cortisol levels. Activation of the HPA axis increases cortisol levels and initiates physiological stress responses. HPA axis dysfunction is associated with stress-related mental health issues. Cortisol levels are considered a promising approach to predict suicidal behavior in animals after social stress. Previous studies have indicated a relationship between cortisol levels and clinical depression has been indicated by studies. Plasma cortisol levels are commonly used as a measure of biologically active cortisol, which is influenced by social and environmental factors, such as social isolation. The findings of this study align with previous research in stressed rats experiencing elevated cortisol levels.

INDCA showed dose-dependent prevention of plasma cortisol increase in stress control rats in the present study. However, subacute fluoxetine pretreatment failed to prevent stress-induced elevation of plasma cortisol levels. In fact, fluoxetine-administered rats showed a significant increase in plasma cortisol levels compared to the stress-control rats, a finding that is consistent with previous research. The stress-relieving property of AS from C. asiatica leaves has been reported through HPA axis mediation and normalizing cortisol levels. In addition, C. asiatica leaves extract exhibited neuroprotective effects against stress-induced neuronal atrophy in albino mice under stress. Therefore, the maintenance of normal cortisol levels may largely contribute to the ability of INDCA to ameliorate stress-induced suicidal behavior.

**CONCLUSIONS**

This study demonstrated the ameliorative effects of oral administration of INDCA against stress-induced suicidal behavior-related traits in rats through the prevention of cortisol augmentation.
The results of the present study, along with previous scientific evidence of its antidepressant efficacy, highlighted the potential of INDCA as a natural and safe therapeutic option for stress-induced disorders. However, further clinical studies are warranted.

ACKNOWLEDGMENT
The authors thank Dr. K. G. Bothara, former Principal of Sinhgad Institute of Pharmacy, Narhe, Pune, India, for providing the necessary infrastructure.

Conflict of Interest
The authors declare no conflicts of interest.

Funding Sources
The study was supported by Indus Biotech Limited, Pune, India (Grant Project No: IBS317).

REFERENCES
20. D’Elia ATD, Juruena MF, Coimbra BM, Mello...


43. Takahashi A. The role of social isolation stress in escalated aggression in rodent models. *Neurosci Res*. 2022;In Press.


63. Liu Q, Cole DA. The association of phasic irritability (aggressive outbursts) and tonic irritability (irritable mood) to depression occurrences, symptoms, and subtypes. *J Affect Disord.* 2021;293:9-18.


70. Moradi K, Badipour A, Moradi A, Bagheri S, Soltani ZE, Moassefi M, et al. Sumatriptan attenuates fear-learning despair induced by


