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ORIGINAL ARTICLE

Tinospora cordifolia Ameliorates Behavioral Deficits in Conditioned Fear and Single Prolonged Stress–Induced Preclinical PTSD Model in Mice by Modulating Translocator Protein (18kDa, TSPO)

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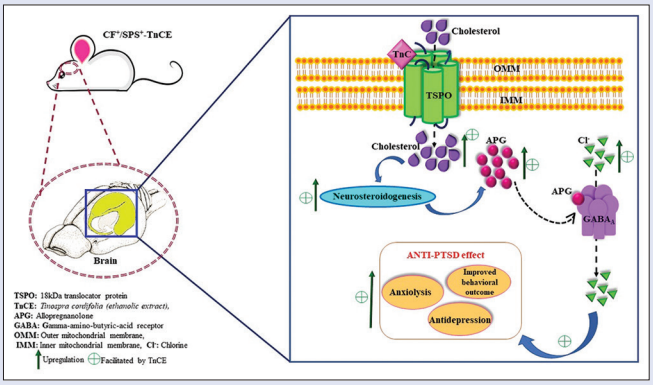
ABSTRACT

Background: Post-traumatic stress disorder (PTSD) is a severe chronic psychiatric condition for which currently there is no specific therapy. Translocator protein (18 kDa; TSPO), a critical therapeutic target for treating PTSD and other neurological deficits regulates allopregnanolone biogenesis. Allopregnanolone potently mediates allosteric modulation of $GABA_A$ which subsequently coordinates emotional behavior. The well-known ayurvedic plant *Tinospora cordifolia* is rich in therapeutically active phytoconstituents which contribute toward its diversified efficacy against neurological disorders like anxiety, depression, etc. **Materials and Methods:** In the present study, we explored the potency of *Tinospora cordifolia* in modulating TSPO to improve PTSD symptomatology in preclinical mice model of combined model of conditioned fear (electric foot shock) and single prolonged stress to induce PTSD. A series of behavioral assessments, histopathological investigations, ELISA and Western blot analysis were conducted to decipher a potential molecular anti-PTSD mechanism of ethanolic extract of *Tinospora cordifolia* extract TnCE. **Results:** Following the treatment protocol, TnCE revealed prominent anxiolytic and antidepressant activity in PTSD-afflicted mice with simultaneous improvement of social behavior and attenuated context memory. Interestingly, the positive impact of TnCE was completely abolished by TSPO selective antagonist PK11195. Moreover, consistent upregulation of TSPO expression with a marked escalation in APG and GABA levels indicated a TSPO-dependent mechanism underlying the pharmacotherapeutic efficacy of TnCE in the PTSD model. **Conclusion:** Thus, this multi-faceted beneficial TnCE may offer a novel therapeutic entity for PTSD treatment by modulating TSPO mediated allopregnanolone biogenesis.

Key words: Allopregnanolone, CF+SPS, PTSD, *Tinospora cordifolia*, TSPO

SUMMARY

- PTSD, recognized as a chronic and debilitating psychiatric disorder, is still lacking any specific therapy. *Tinospora cordifolia* is widely known for its ameliorative effects against various psychological conditions. In the current study, we examined the anti-PTSD efficacy of *T. cordifolia* in Swiss albino mice and also explored its probable molecular mechanism. Our results indicated that TSPO (translocator protein, 18 kDa) expressed in the outer mitochondrial membrane upon activation by TnCE enhances neurosteroidogenesis of APG. The increased level of APG in turn promotes modulation of $GABA_A$ mediated neurotransmission that confers in improved behavioral adaption to PTSD.



Abbreviations used: TSPO: 18 kDa Translocator Protein; TnCE: *Tinospora cordifolia* ethanolic extract; APG: Allopregnanolone; GABA: gamma-amino-butyric-acid receptor.

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INTRODUCTION

A multifarious psychiatric condition, post-traumatic stress disorder (PTSD), owing to its soaring global prevalence among the general populace, has been referred by the Global Burden of Diseases report as one of the primary contributors of worldwide disease burden.[1,2] PTSD is recognized as a chronic and debilitating psychiatric disorder by the American Psychiatric Association, DSM-IV in which an individual suffers from incessant reexperiences of intrusive and tormenting reminiscence of traumatic past events.[3]

However, any definite mechanism regulating the complex psychological and behavioral outcomes of PTSD remains to be explored.

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Neurosteroidogenesis has recently been observed to play a critical role in PTSD pathophysiology. The search for biological markers has led to one of the current consensuses of altered biosynthesis of allopregnanolone, a neurosteroid, to be intricately associated with PTSD.[4] Truncated allopregnanolone (APG) levels in individuals suffering from PTSD is actively responsible for misbalancing the inhibitory and excitatory neurotransmission cascade that escalates PTSD recollections as well as depressive symptoms. As revealed from earlier studies, corticolimbic APG content positively correlates to anxiolytic effect and abates aggression.[5] Selective serotonin reuptake inhibitors (SSRIs), paroxetine and sertraline (FDA approved), which are first line therapeutics for core symptoms of PTSD, act as selective brain steroidogenic stimulants (SBSSs) and particularly increase APG biosynthesis. This in turn potentially modulates gamma-amino-butyric-acid receptor ($GABA_A$) neurotransmission.[6,7]

On the corollary, the quest for new therapeutic targets has also revealed translocator protein (18 kDa) (TSPO), primarily expressed by glial cells to play a pivotal role in the treatment of neurological ailments such as PTSD.[8,9] Peripheral tissue as well as the central nervous system possess TSPO in the outer mitochondrial membrane that regulates neurosteroid production from cholesterol and their availability in the brain.[10] TSPO is thus being considered to potentially improve depression and anxiety by inducing neurosteroid biogenesis.

Current pharmaco-treatment of PTSD relying on SSRIs is associated with detained onset of action, serious side effects, non-responsiveness or partial response and other shortfalls,[11] which necessitates novel therapeutic modalities against PTSD symptoms. With the recent advancement in the field of phytoconstituents, a wide spectrum of biomoieties have been identified to exhibit promising effects in psychiatric disorders with additional advantages of cost efficiency and minimal side effects. *Tinospora cordifolia* (synonym: *Tinospora sinensis* (Lour.) Merr.)) is also known as giloya, guduchi, and amrita. *Tinospora cordifolia* (TnC), a well-known ayurvedic medicine, is categorized as adaptogen or rejuvenator that aids in improvement of memory and cognition.[12] Ethanolic extract of TnC (TnCE) upon oral administration has shown promising activity in alleviation of oxidative stress with simultaneous betterment of locomotor activity.[13] Reversal of depression-like state has also been reported in mice.[14] TnC is thus acclaimed to be effective in maintenance of healthy and proper functionalities of the brain as well as in stress management.[12,15]

Based on these observations, we hypothesized TnC to be efficient in modifying stress-induced psychiatric conditions like PTSD. Also, the pharmacotherapeutic effect of TnC as a modulator of TSPO has also not been explored in preclinical PTSD mice model. Thus, in the current study, we aimed at investigating the therapeutic potency of TnCE in conditioned fear (CF) + single prolonged stress (SPS) mice model of PTSD,[16] and explore its mechanistic role.

MATERIALS AND METHODS

Extraction of TnC

The stem barks of TnC were collected in dried powdered form (HM Herbals, Ranchi, India; Ref No. HM/972) and primarily authenticated by Dr S. Jha (Professor, Dept of Pharmaceutical Sciences and Technology, BIT, Mesra). Initially, the dried powder was subjected to defatting for removal of fats and lipids followed by subsequent extraction with 98% v/v ethanol using Soxhlet apparatus (68°C–78°C) for 24 hr. After filtering the extract using Whatman (grade 1) filter paper, a rotary evaporator was used to evaporate the solvent at 40°C under reduced pressure. TnCE (16.7% w/w, yield) was stored at 4°C for further use.[17] The extract was also validated through preliminary phytochemical screening which revealed the presence of alkaloids, glycosides, flavonoids, saponins, steroids, carbohydrates, tannins and proteins, as reported earlier.[14]

Animals

Seven-week-old male Swiss albino mice (n = 8) were obtained and housed in well-aerated polystyrene cages at optimum temperature and humidity-controlled environment with 12-hr light/dark cycles. The animals were provided with free access to standard pelleted diet and water *ad libitum*. All the experiments were performed with prior approval from the Institutional Animal Ethical Committee, BIT Mesra (1972/PH/BIT/96/20/IAEC) in accordance with CPCSEA guidelines.

CF-SPS protocol to induce PTSD symptomatology in mice

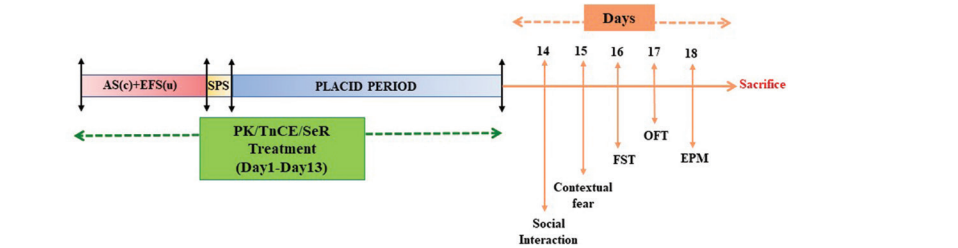
The single prolonged stress (SPS) model combined with conditioned fear (CF) technique was executed following a previously described method.[16,18] Briefly, the experimental animals were trained daily by delivering electric foot shock (EFS) for five days. Following 60 secs of the adaptation period, each mouse was exposed to 10 secs of auditory cue (80 db of white sound). During the final 2 secs of the cue signals, 1 mA EFS was delivered to the respective animals. On the 6th day, the mice were subjected to SPS and primarily immobilized for 2 hr preceding forced swimming for 10 mins and then 15 min exposure in a beaker soiled with beddings from sentinel rat cages. The final step involved gradual loss of consciousness of the experimental rodents by inhalation of diethyl ether. All animals were kept undisturbed for the succeeding seven days in home cages with fresh bedding.

Experimental design

Mice were randomly divided into six groups (n=8). While group I comprised of non-stressed animals (CF^-/SPS^-), group II animals (CF^+/SPS^+) were exposed to CF + SPS and treated only with vehicle. Mice in groups III and IV (CF^+/SPS^+–$TnCE_{100/200}$) were administered with TnCE at 100 mg/kg, b.w.; p.o. and 200 mg/kg b.w.; p.o., respectively. Group V (CF^+/SPS^+–SeR) received sertraline (SSRI) (SeR [Sigma Aldrich]; 15 mg/kg, b.w.; p.o.), and group VI (CF^+/SPS^+–PK + TnCE) animals were pretreated with PK11195 (a selective TSPO antagonist [Sigma-Aldrich]; 3 mg/kg, b.w.; i.p.) 30 min before TnCE (200 mg/kg b.w; p.o.) treatment. The treatments were simultaneously continued during 13 days of CF + SPS protocol. On and from the 14th day, different behavioral procedures were performed [Figure 1]. Two-and-a-half hours succeeding the elevated plus maze test, the mice were sacrificed via cervical dislocation and brains were carefully dissected after proper perfusion. The hippocampal tissues were isolated and preserved accordingly for histopathology, ELISA, and Western blot analysis.

Social interaction test

On day 14, individual experimental animals were subjected to the three-chamber social interaction test by introducing them in a central chamber provided with the option of either communion with another mouse placed under a small wired cup (social) or empty wired cup in the other chamber.[19] Preceding placement of the novel mouse (matched to the corresponding strain, age, weight of the test mice) in the wired cup of the side chamber, the test animals were habituated in the middle chamber for 10 mins and then the chamber separator was removed. Behavioral parameters assessment included estimations of total distance traversed, number of entries in the area nearby the cup containing the interacting mouse as well as in the chamber holding the stranger mouse along with their corresponding time in cups or arena.



Groups	Intervention	Dose
Group I (CF^-/SPS^-)	Normal control animals (no treatment)	--
Group II (CF^+/SPS^+)	Induced control animals subjected to conditioned fear (CF) +single prolonged stress (SPS)	--
Group III CF^+/SPS^+-$TnCE_{100}$	PTSD inflicted animals treated with *Tinospora cordifolia* (ethanolic extract)	100 mg/kg b.w; p.o.
Group IV CF^+/SPS^+-$TnCE_{200}$	PTSD inflicted animals treated with *Tinospora cordifolia* (ethanolic extract)	200 mg/kg b.w; p.o.
Group VCF+/SPS+-SeR	PTSD inflicted animals treated with sertraline (SSRI)	15mg/kg, b.w.; p.o
Group VI (CF^+/SPS^+-PK+TnCE)	PTSD inflicted animals were pretreated with PK11195 (a selective TSPO antagonist; 30 minutes before TnCE	PK11195: 3 mg/kg, b.w.; i.p. TnCE: 200 mg/kg b.w; p.o.

AS(c): Auditory stimulus (conditioned stimulus)
EFS(u): Electric foot shock (unconditioned stimulus (1mA)
FST: Forced swim test
OFT: Open field test
EPM: Elevated plus maze

Figure 1: Experimental design representing conditioned fear + single prolonged stress–induced PTSD model in Swiss albino mice for evaluation of efficacy of TnCE treatment evaluated through (a) social behavior test, (b) contextual fear conditioning, (c) forced swim test, (d) open field test and (e) elevated plus maze. The 6 experimental groups used in this study has also been depicted ($n = 8$). AS (c): Auditory stimulus (as conditioned stimulus), EFS (u): Electric foot shock (as unconditioned stimulus (1 mA), CF: Conditioned fear; SPS: Single prolonged stress (restrain + forced swim test + predator betting scent + diethyl ether inhalation), TnCE: *Tinospora cordifolia* (ethanolic extract); SeR: Sertraline

Contextual fear analysis

The rodents upon re-exposure to the shock chamber exhibit intermittent freezing and this behavior is correlated with fearful memory induced by trauma associated with a specific context. Thus, measurement of contextual freezing is an effective method for evaluation of PTSD.[11] For estimation of contextual fear, on day 15, the experimental animals were returned to the same shock chamber that served as recapitulation of the previous foot shock session for 5 mins. Total cumulative freezing time and activities were video recorded to be analyzed later (Ethovision ver 3.0; Noldus Technology).

Forced swim test

The forced swim test was performed according to the slightly modified technique of Porsolt *et al.*[20] Briefly, glass cylinders (25 cm × 10 cm) were filled with Room Temperature (RT) water (25°C) and the experimental mice, on day 16 were subsequently left inside these for 6 min and allowed to swim. After initial vigorous activity for 2 min, the mice underwent an immobile state and this duration of immobility (4 min) was compared among the various experimental animals. The experimental sessions were videotaped (Ethovision ver 3.0, Noldus Technology) for analysis of immobility.

Open field test

In order to investigate the possibility of contextual freezing reversion in PTSD mice by TnCE administration being interrelated with alterations in locomotor activity, we performed the open field test.[21,22] On the 17th day, spontaneous locomotion was determined by introducing the mice in a Plexiglas box of dimensions 36 × 29 × 23 cm for 5 mins. The base of the box was properly subdivided into equal square sections and the animals were placed carefully at its center. Distance covered in the open field along with a total number of crossings (when all the 4 paws were inside a new box) and rearing (front paws when lifted together from the floor) were counted by an observer blind to the experimental study design. The interiors of the walls and floor of the box were thoroughly wiped with ethyl alcohol for avoiding the consequences of smell and faeces between experimental mice.

Elevated plus maze test

On the 18th day, the animals were subjected to elevated plus maze, a test for evaluating PTSD intertwined anxiogenic behaviors in animals.[11] The maze was elevated at a height of 40 cm from the ground with the two opposite-sided open arms (lacking walls) and two dark-walled enclosed arms being connected by a central common platform (5 × 5) cm^2. The test mouse was placed on the central platform of the maze such that it was faced towards the dark closed arms. The number of entries in both open and closed arms (30 cm × 5 cm) were documented only when all four paws of the mouse were on the arm base with simultaneous recording of the time elapsed in corresponding arms. Sessions were taped using Ethovision version 3.0 (Noldus Technology). While the ratio of open arm entries to total arm entries represented the percentage of open arm entries, the percentage of time in open arms were calculated by dividing time elapsed in open arms by total time in both arms.[23]

Histopathological examination of hippocampal tissue

The isolated hippocampal tissue from all experimental animals were subjected to standard hematoxylin and eosin (H&E) staining protocol for histopathological examinations.[24] Briefly after graded dehydration, the tissues were subjected to xylene vitrification and embedded in paraffin. Then after subsequent dewaxing and rehydration, H and E stained tissues were observed under Leica DM E microscope.

Estimation of allopregnanolone and GABA by ELISA

The extraction of hippocampal tissues dissected after sacrifice was carried out on ice using the lysis buffer containing NaCl (137 mM), sodium vanadate (0.5 mM), aprotinin (10 µg/mL), leupeptin1% NP40 (1 µg/mL), glycerol (10%), PMSF (1 mM), and Tris-HCl (20 mM; pH 8.0). The concentration of allopregnanolone (Arbor Assays, United States) and GABA (Novatein Biosciences, Cambridge MA) were determined with the help of commercially available ELISA kits following the manufacturer's instructions.

Western blot analysis of TSPO

The isolated hippocampal tissue preserved in liquid nitrogen was washed thoroughly thrice with phosphate-buffered saline (PBS), and then lysed for 10 min with ice-cold 500 µL of lysis buffer that constituted of NaCl (50 mM), sucrose (0.5M), HEPES (10 mM; pH 7.9), EDTA (0.1 mM), triton (0.5%), DTT (1 mM), phenylmethylsulfonyl fluoride (PMSF; 1 mM), protease inhibitor (5 µL), and phosphatase inhibitor (5 µL) cocktails. Then cell lysates were centrifuged (10 min, 1000 rpm) at 4°C which was successively followed by Bradford protein assay for quantifying protein.[24] Then, SDS-PAGE was conducted and the separated proteins were carefully transferred electrophoretically to nitrocellulose membrane (Millipore). The non-specific sites of the protein blots were blocked by incubating for 1 hr with non-fat dry milk in Tris-buffered saline and Tween 20 mixture (TBST; 5% w/v) at room temperature. Then, after overnight incubation (4°C) of blots with primary antibodies, the blots were thoroughly washed thrice with TBST (each for 5 min). Re-incubation of the blots was then performed for 1 hr (RT) using 1:20,000 dilution of HRP-conjugated secondary antibody. This was followed by developing membranes with chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA, USA) and then acquired on film (CL-XPosure, 8 × 10 in). The following primary antibodies of TSPO and β-actin (Cell Signaling Technology, Danvers, MA, USA) was used at a predetermined dilution (1:500 in 5%w/v BSA in TBST). Image J software (NIH, Washington, USA) was employed for densitometry estimation.[25]

Statistical analysis

Unpaired student's *t*-tests were carried out for the determination of social behavior in social interaction test. For all the other behavioral studies, one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison tests were used for statistical comparisons of the data among the different experimental groups of animals with similar sample size. The level of significance was measured at $P < 0.05$. All data were presented as mean ± standard error of the mean (SEM).

RESULTS

TnCE improved the social behavior in CF$^+$/SPS$^+$ mice

The outcome of the social behavioral test is depicted in Figure 2. The SPS$^-$/CF$^-$ animals in group I exhibited preference for the chamber holding the novel mice, thus indicating regular social behavior. A higher number of entries was observed in both the cup area ($P < 0.01$; Figure 2a.i) as well as the arena surrounding the novel mice holding cup ($P < 0.001$, Figure 2b.i), while the time spent in both the occupied cup area [Figure 2a.ii] and near the arena [Figure 2b.ii] was observed to be significantly high ($P < 0.001$) among the CF$^-$/SPS – mice in the social chamber. However, deterioration of this normal social behavior was evident among group II animals exposed to CF + SPS as the difference in number of entries and time spent [Figure 2a.i and 2a.ii respectively] in the occupied cup area was insignificant ($P > 0.05$) to that of the empty cup while maintaining inclination for social arena ($P < 0.05$) in case of both number of entries [Figure 2b.i] and time spent [Figure 2b.ii]. A dose-dependent effect of TnCE was observed upon the social behavior. Effective in 100 mg/kg, b.w., p.o, (group III), TnCE when administered in dose 200 mg/kg, b.w. (p.o.) in group IV animals, it significantly increased ($P < 0.01$) their number of entries and time spent in the cup area holding the novel mouse and also the arena surrounding it with respect to the empty cup, which was in parallel to the response of SeR treatment in group V mice. However, PK11195 pre-treatment completely blocked the behavioral improvements effectuated by TnCE and the mice in group VI not only failed to regain interest for the social cup area but also lost the proclivity for the social arena. However, total distance travelled by the mice [Figure 2c] in different experimental groups were found to be statistically insignificant ($P > 0.05$).

TnCE alleviated the context mediated conditioned fear in CF$^+$/SPS$^+$ mice

Fear memory evaluated by the freezing intensity to contextual cue is represented in Figure 3a. In comparison to unstressed mice in group I, exposure to context markedly escalated the freezing response ($P < 0.001$) in CF$^+$/SPS$^+$ animals (group II) [Figure 3a]. TnCE treatment in both the doses (100 and 200 mg/kg, b.w., p.o) reduced the freezing time with the higher dose showing maximum potency ($P < 0.01$) relative to group II stressed animals. Similarly, SeR also significantly decreased the freezing time ($P < 0.001$) in group V animals. However, PK11195 markedly antagonized the effect of TnCE treatment in group VI and exponentiated the freezing reaction of the stressed animals ($P < 0.001$). Such results imply that mice exposed to traumatic situations related to context suffer from experiencing incessant fear response, reflected as freezing behavior, which can be alleviated following repeated TnCE administration and may have partially been mediated by elevated TSPO expression and APG upregulation.

TnCE exhibited antidepressant-like activity in forced swim test among the CF$^+$/SPS$^+$ mice

The group II animals exposed to CF + SPS developed PTSD symptomatology, as indicated by the increment in time of immobility ($P < 0.001$) in forced swim test relative to that of SPS$^-$/CF – animals [Figure 3b]. Chronic administration of TnCE reduced this immobility in a dose-dependent fashion. While 100 mg/kg, b.w., p.o, dose of TnCE caused reduction in the immobility time ($P < 0.05$), when administered at a dose of 200 mg/kg, b.w., p.o, group IV animals showed marked improvement and the immobility was further minimized ($P < 0.01$) in comparison to CF$^+$/SPS + mice (group II). This effect of TnCE was also compared with the antidepressant activity of SeR. However, this TnCE-mediated decline in mobility was reversed ($P < 0.01$) by TSPO antagonist PK11195 in group VI animals, suggesting activation of TSPO to be partly responsible for mitigating PTSD like symptoms.

TnCE does not impair spontaneous locomotion

The consequence of TnCE treatment on spontaneous locomotion was explored by the open field test. As revealed in Figure 3c, there was a non-significant effect ($P > 0.05$) on distance travelled [Figure 3c.i], a total number of crossings [Figure 3c.ii] and rears [Figure 3c.iii] between the animal groups. These results indicated that neither repeated doses of TnCE nor SeR or PK11195 adversely affected the locomotor activity and that TnCE treatment ameliorates contextual fear unbiased of locomotory changes.

Anti-anxiety-like effect of TnCE in CF$^+$/SPS + mice

Animals in group II subjected to stress demonstrated a significant decrease ($P < 0.001$) in percentage of both entries [Figure 4a.iii] and time spent [Figure 4a.iv] in open arms of elevated plus maze with respect to animals belonging to (SPS$^-$/CF$^-$) group I animals, thus confirming anxiety-induced behavior of the PTSD model. In contrast, repeated administration of TnCE (in groups III and IV) significantly enhanced both the parameters with the maximum response at 200 mg/kg, b.w., p.o in group IV animals ($P < 0.001$). Similarly, SeR treatment was also found to increase the percentage of entries and time elapsed ($P < 0.001$) in open arms in comparison to the CF$^+$/SPS + group of mice. But pre-treatment with TSPO antagonist PK11195 impeded ($P < 0.001$) the anxiolytic

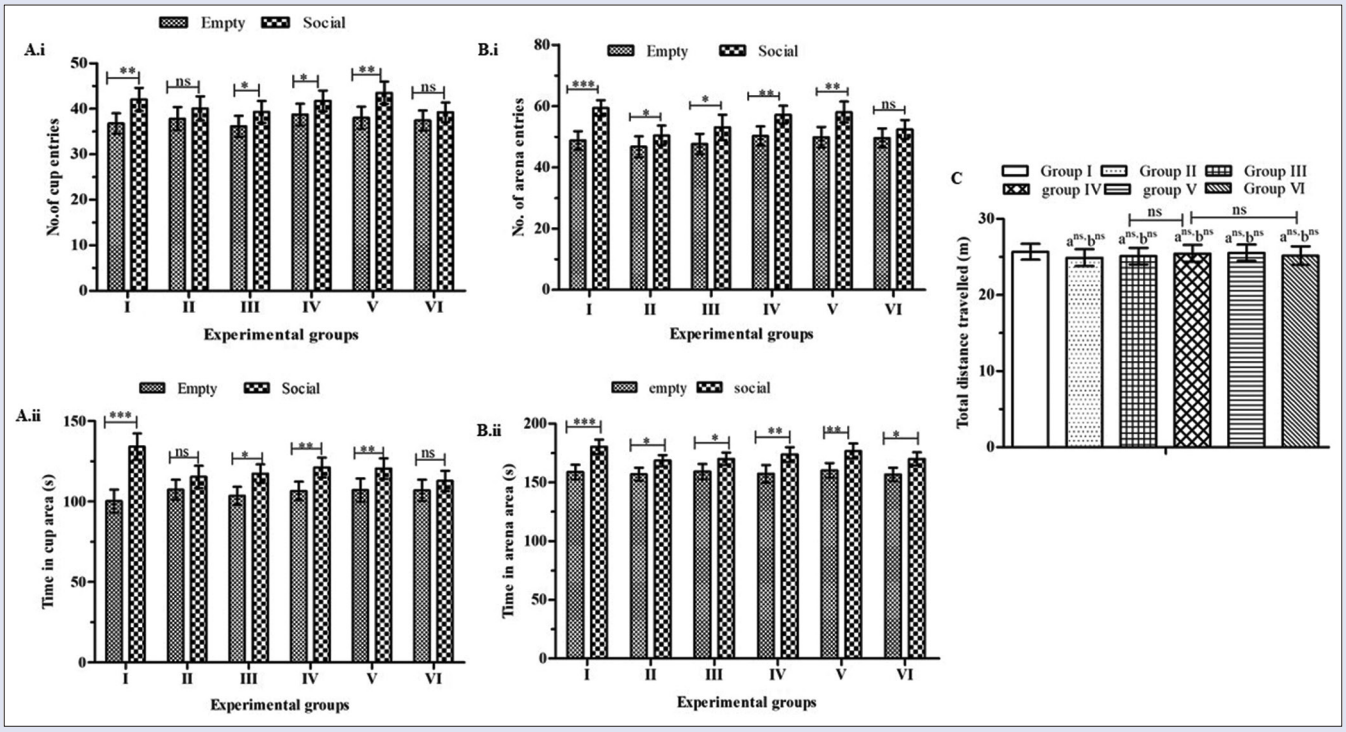


Figure 2: Social interaction effects of TnCE treatment in CF+SPS stress-induced animal. 2A (i and ii) represents comparison between number of entries as well as time elapsed in area of empty and occupied cup; 2B (i and ii) represents comparison between number of entries as well as time elapsed in arena surrounding empty and occupied cup; data are represented as the mean ± SEM ($n = 8$). Unpaired *t*-test was employed for the assessment of sociability within each group; 2c represents total distance travelled by animals. One-way ANOVA followed by Bonferroni test was used for analysis by comparing groups III, IV, V, VI with group II, Significant: $^{ns}P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{ns}P > 0.05$ when group IV is compared with group III, $^{ns}P > 0.05$ when group VI is compared with group IV; Group I: CF^{-}/SPS^{-} (No stress + vehicle), Group II: CF^{+}/SPS^{+} (stress + vehicle), Group III: CF^{+}/SPS^{+}–$TnCE_{100}$ (stress + TnCE treatment, 100 mg/kg, b.w., p.o); Group IV: CF^{+}/SPS^{+}–TnC_{200} (stress + TnC treatment, 200 mg/kg, b.w., p.o), Group V: CF^{+}/SPS^{+} –SeR_{15} (stress + SeR, 15 mg/kg, b.w., p.o), Group VI: CF^{+}/SPS^{+}–PK + TnCE [stress + PK11195 (3 mg/kg, b.w.; i.p)+TnCE (200 mg/kg, b.w., p.o)], SPS: single prolonged stress, CF: Conditioned fear, TnCE: *Tinospora cordifolia* (ethanolic extract); SeR: Sertraline, APG: Allopregnanolone, TSPO: Translocator protein, PK11195: TSPO antagonist

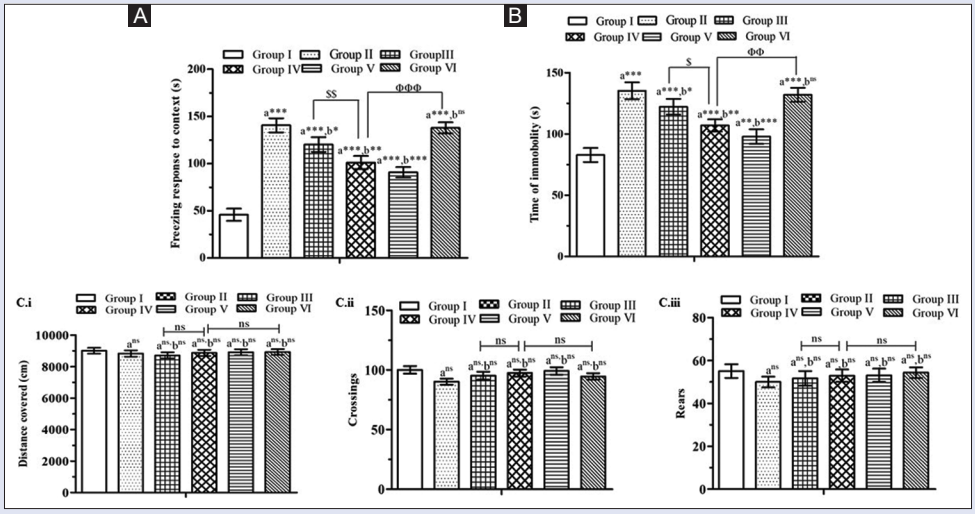


Figure 3: Effects of TnCE extract administration on (A) CF + SPS–induced conditioned fear response to context exposure, (B) Immobility time in forced swimming test, (C) on locomotor activity (open field test) measured in terms of (i) distance travelled crossings, (ii) crossings and (iii) rear. Data are represented as the mean ± SEM, ($n = 8$), Comparisons: a = group II, III, IV, V, VI with group I and b = group III, IV, V, VI with group II, Significant: $^{ns}P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{\$}P < 0.05$; $^{\$\$}P < 0.01$ when group IV compared with group III; $\varphi\varphi\varphi P < 0.001$ when group VI compared with group IV; Group I: CF^{-}/SPS^{-} (No stress + vehicle), Group II: CF^{+}/SPS^{+} (stress + vehicle), Group III: CF^{+}/SPS^{+}–$TnCE_{100}$ (stress + TnCE treatment, 100 mg/kg, b.w., p.o); Group IV: CF^{+}/SPS^{+}–TnC_{200} (stress + TnC treatment, 200 mg/kg, b.w., p.o), Group V: CF^{+}/SPS^{+} –SeR_{15} (stress + SeR, 15 mg/kg, b.w., p.o), Group VI: CF^{+}/SPS^{+}–PK + TnCE [stress + PK11195 (3 mg/kg, b.w.; i.p)+TnCE (200 mg/kg, b.w., p.o)], SPS: single prolonged stress, CF: Conditioned fear, TnCE: *Tinospora cordifolia* (ethanolic extract); SeR: Sertraline, APG: Allopregnanolone, TSPO: Translocator protein, PK11195: TSPO antagonist

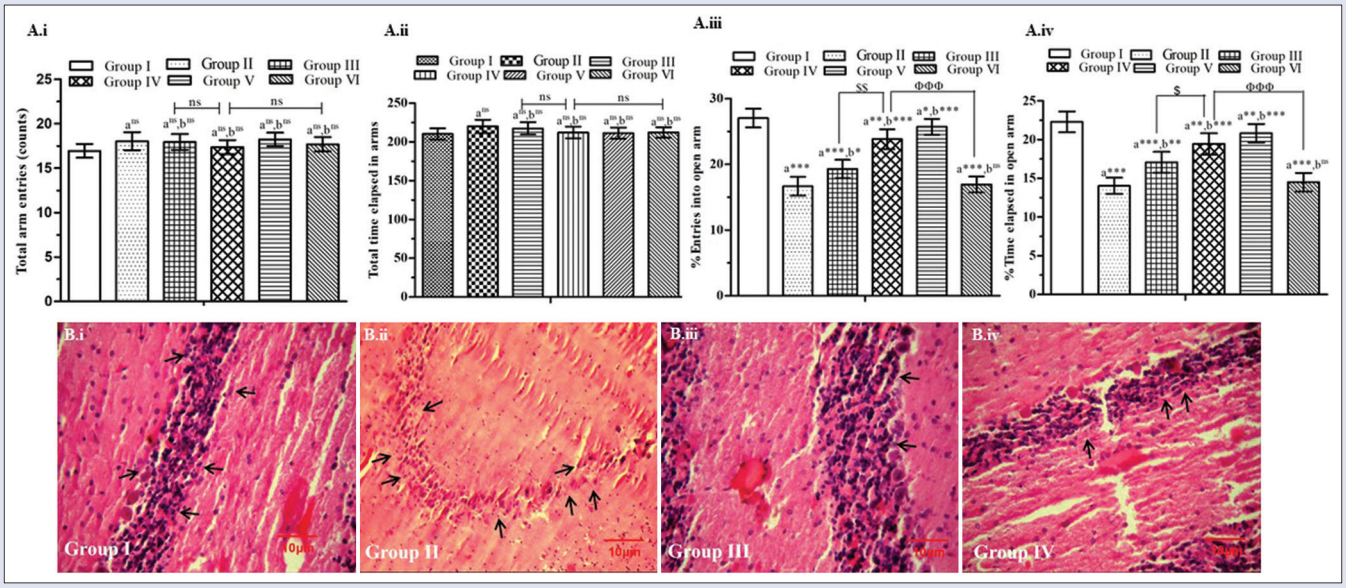


Figure 4: (A) Anxiolytic-like potency of chronic TnCE treatment in CF + SPS–induced mice as quantified by (i) total arm entries, (ii) total time elapsed in arms, (iii) % entries into open arms (iv) % time elapsed in open arms in the elevated plus maze. Data are represented as mean ± SEM, ($n = 8$), Comparisons: a = group II, III, IV, V, VI with group I and b = group III, IV, V, VI with group II, Significant: $^{ns}P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{\$}P < 0.05$; $^{\$\$}P < 0.01$ when group IV compared with group III; $\varphi\varphi\varphi P < 0.001$ when group VI compared with group IV; (B) Photomicrographs representing histopathological analysis of hippocampal tissue of animals in (i) group I, (ii) group II, (iii) group III and (iv) group IV. Arrows indicate microstructural changes. Group I: CF^{-}/SPS^{-} (No stress + vehicle), Group II: CF^{+}/SPS^{+} (stress + vehicle), Group III: CF^{+}/SPS^{+}–$TnCE_{100}$ (stress + TnCE treatment, 100 mg/kg, b.w., p.o); Group IV: CF^{+}/SPS^{+}–TnC_{200} (stress + TnC treatment, 200 mg/kg, b.w., p.o), Group V: CF^{+}/SPS^{+} –SeR_{15} (stress + SeR, 15 mg/kg, b.w., p.o), Group VI: CF^{+}/SPS^{+}-PK + TnCE [stress + PK11195 (3 mg/kg, b.w.; i.p)+TnCE (200 mg/kg, b.w., p.o)], SPS: single prolonged stress, CF: Conditioned fear, TnCE: *Tinospora cordifolia* (ethanolic extract); SeR: Sertraline, APG: Allopregnanolone, TSPO: Translocator protein, PK11195: TSPO antagonist

efficacy of TnCE. However, in cases of total arm entries [Figure 4a.i] and total time elapsed in all arms [Figure 4a.ii], the groups exhibited insignificant differences. Such results elaborated efficient amelioration of anxiogenic like behavior in animals upon exposure to combined stressors by TnCE which may partly be dependent on TSPO and APG coordinated mechanisms.

TnCE upon chronic administration restored the hippocampal microarchitecture of CF^{+}/SPS^{+} animals

The photomicrographs represent the variations in tissue architecture of the hippocampus. When closely observing, unstressed animals in group I demonstrated normal neurons showing usual cytoplasmic arrangements and distinct nuclear structure [Figure 4b.i]. In contrast, the PTSD-afflicted mice revealed marked neurodegeneration with deformed and shrunk nucleus in the *cornu Ammonis* region of the hippocampus [Figure 4b. ii]. However, chronic administration of TnCE demonstrated restorative potential [Figure 4b.iii], as with its increasing dose the neuronal abrasions and deformities were significantly reduced and 200 mg/kg, b.w. p.o dose of TnCE showed maximum protection [Figure 4b.iv].

Repeated administration of TnCE increased APG and GABA concentration in hippocampal tissue

As exhibited in Figure 5a, the APG concentration prominently decreased ($P < 0.001$) in group II (CF^{+}/SPS^{+}) animals relative to the unstressed mice in group I. Concurrently, the level of GABA was also depleted remarkably ($P < 0.001$) in the PTSD induced animals [Figure 5b]. However, chronic oral administration of TnCE successfully resulted in a dose-dependent enhancement of APG as well as GABA concentration. Animals in groups IV and V receiving higher dose of TnCE and SeR, respectively, exhibited maximum increment in the neurosteroid (APG) concentration ($P < 0.001$) in comparison to group II stressed animals, which consequently improved ($P < 0.001$) the GABA concentration also. Contrary to this, the restored APG level by TnCE treatment was evidently truncated ($P < 0.001$) by TSPO antagonist PK11195 which was reciprocated in the GABA levels exhibiting significant ($P < 0.01$) reduction in group VI animals [Figure 5a and b]. Thus, ELISA confirmed the reversal of PTSD-like symptoms by TnCE to be partly regulated by TSPO mediated escalation of neurosteroid biogenesis in the brain via sequential increment of GABA.

TnCE treatment augmented TSPO expressions in CF^{+}/SPS^{+} mice

Group II mice that developed PTSD symptomatology induced by CF + SPS revealed downregulated expression of TSPO ($P < 0.001$) in the hippocampus of the brain relative to that in group I (CF^{-}/SPS^{-}) mice [Figure 5c]. However, when compared to group II animals, daily TnCE administration (200 mg/kg, b.w., p.o.) elevated TSPO ($P < 0.001$) expression, thereby facilitating its anti-PTSD effect. In contrast, PK11195 pre-treatment blocked the ameliorative effects of TnCE which was reflected in the behavioral studies, thereby validating the involvement of TSPO-mediated APG-induced GABAergic neurotransmission as a probable pathway for the activity of TnCE.

DISCUSSION

Recent progress in the field of herbal psychopharmacology has unveiled sundry of promising biomoieties beneficial for treating anxiety and stress. Various extracts of TnC and its polyherbal formulations exhibit neuroprotective functions. While aqueous, methanolic and petroleum ether extracts manifest improvement in memory, anti-anxiogenic and antidepressant potential,[13] ethanolic root extract is reported to be effective in stress management and healthy functioning of the brain.[26] It

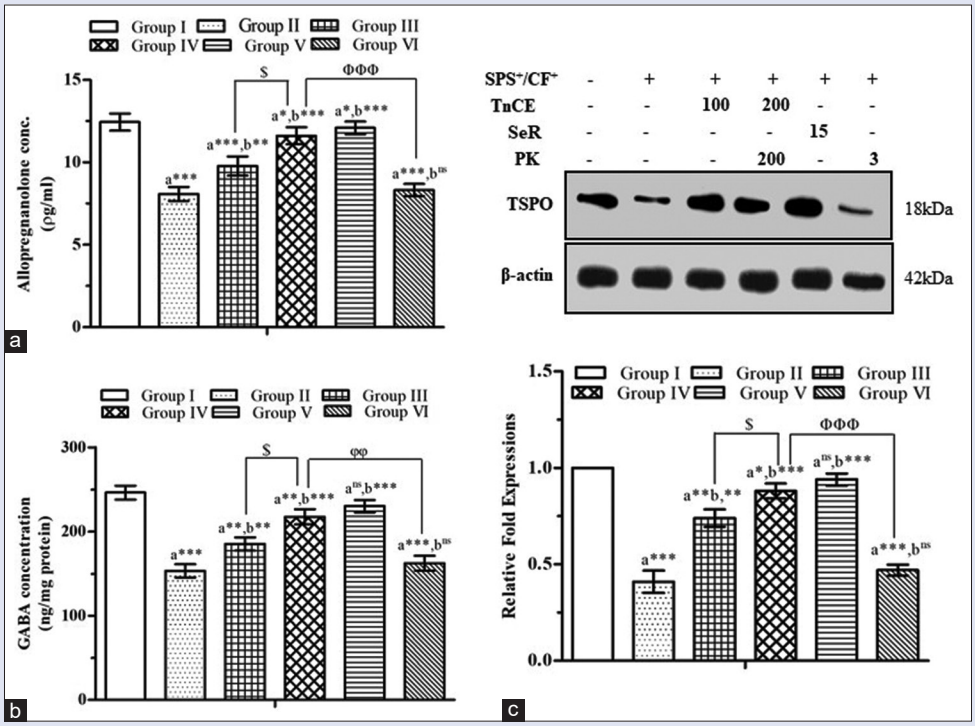


Figure 5: ELISA analysis of (a) APG concentration (b) GABA concentration analysis in the hippocampal brain tissue, (c) Western blot analysis of TSPO (18 kDa protein) of the hippocampal brain tissue. Data are represented as the mean + SEM, ($n = 8$), Comparisons: a = group II, III, IV, V, VI with group I and b = group III, IV, V, VI with group II, Significant: $^{ns}P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{\$}P < 0.05$; $^{\$\$}P < 0.01$ when group IV compared with group III; φφφ$P < 0.001$ when group VI compared with group IV; Group I: CF^{-}/SPS^{-} (No stress + vehicle), Group II: CF^{+}/SPS^{+} (stress + vehicle), Group III: CF^{+}/SPS^{+}–$TnCE_{100}$ (stress + TnCE treatment, 100 mg/kg, b.w., p.o); Group IV: CF^{+}/SPS^{+}–TnC_{200} (stress + TnC treatment, 200 mg/kg, b.w., p.o), Group V: CF^{+}/SPS^{+} –SeR_{15} (stress + SeR, 15 mg/kg, b.w., p.o), Group VI: CF^{+}/SPS^{+}–PK + TnCE [stress + PK11195 (3 mg/kg, b.w.; i.p)+TnCE (200 mg/kg, b.w., p.o)], SPS: single prolonged stress, CF: Conditioned fear, TnCE: *Tinospora cordifolia* (ethanolic extract); SeR: Sertraline, APG: Allopregnanolone, TSPO: Translocator protein, PK11195: TSPO antagonist

also possesses potent anti-anxiety efficacy and simultaneously improves behavioral deficits in terms of cognition and motor coordination.[27] However, the efficacy of TnC in reversing PTSD condition has still not been investigated, which was the primary aim of the current study. We adopted CF+SPS provoked PTSD model which replicates key symptoms of PTSD resembling anxiety, depression, hyperarousal, and escalated conditional fear[19] in the present study to decipher the probable anti-PTSD mechanism of TnCE.

A battery of behavioral tests was performed in the current study to analyze the pharmacotherapeutic potency of TnCE in alleviating PTSD-like symptoms. Stress-triggered impairment in sociability, accentuated fear response to conditioned stimuli, anxiogenic and depression-like psychiatric behavior elicited by the PTSD inflicted mice in social interaction assessment, contextual fear analysis, elevated plus maze, and forced swim test respectively affirmed the credibility of CF+SPS protocol to establish preclinical PTSD model. However, our results depicted that CF+SPS-mediated anxiogenic or freezing behavior had no influence on the spontaneous locomotion; this suggested that the freezing response to aversive foot shock associated context in PTSD mice was afflicted without altering the locomotory actions that correlate with physiological modifications.[11]

TnCE, as observed in our preliminary phytochemical screening, is also documented to constitute a plethora of active phytochemicals,[28] responsible for imparting diversified neuroprotective effects. While alkaloids like tinosporin, isocolumbin, jatrorhhizine, berberine,[29] choline,[30] hignemanine,[31] palmitine,[32] tembetarine, magnoflorine,[33] isoculombin, tetrahydropalmatine,[34] and aporphine alkaloids are known to improve neurological, psychiatric, anxiety- and depression-like conditions, glycosides like 18norclerodane glucoside, furanoid diterpene glucoside, tinocordiside and tinocordifolioside attenuate ALS, dementia, motor and cognitive deficits.[35] Moreover, 3, (a, 4di hydroxy3methoxybenzyl) 4 (4hydroxy3methoxybenzy l) tetrahydrofuran, Jatrorrhizine Ntransferuloyl tyramine, giloin and tinosporic acid are also used to treat anxiety and depression through inactivation of various neurotransmitters.[36] The presence of such potent bioactive moieties in TnCE may thus signify its observed dose-dependent efficacy in improving social interaction, ameliorating conditioned fear, and facilitating anxiolytic and anti-depressant potency, thereby bating the behavioral deficits of PTSD.

Recently, abnormalities in neurosteroidogenesis are being considered as a major contributor to the pathophysiology of PTSD, which encouraged us to explore the probable underlying mechanism of TnCE in promulgating its anti-PTSD effects. Different clinical trials implied APG, abundantly present in CNS, to be clinically germane in mood disorders such as anxiety and depression.[37–39] This positive allosteric modular of $GABA_A$,[37] has been declared by FDA as the first unique treatment for post-partum depression as endogenous tranquilizers. Plummeted levels of APG on tonic GABA facilitated inhibitory conductance is often associated with the pathophysiology of PTSD, aggression and anxiety.[5,40] Earlier reports indicate the critical role of APG in mediating trauma-associated memory of contextual fear.[4,39] Concurrently, our study also revealed a remarkable reduction in hippocampal APG level in stressed mice which was efficiently revoked following TnCE or SeR treatment. Such results were in agreement with existing knowledge that SSRIs like SeR reverses behavioral despair in psychiatric conditions by augmenting APG synthesis,[2,4,41] while a

number of natural phytoextracts also improve behavioral activities by regulating APG. Additionally, the disrupted expression of inhibitory neurotransmitter GABA in PTSD-afflicted animals was restored to basal levels following TnCE treatment, verifying the potentiation of GABA-regulated neurotransmission. However, it is to be noted that the potential role of other neurosteroids were not considered in the current study and shall be identified in future studies. The present study illustrated the efficacy of TnCE to increase APG biosynthesis via GABAergic modulation, which may partially mediate its anti-PTSD efficacy as reflected in the behavioral studies.

TSPO, which plays a pivotal role in regulating the synthesis of neurosteroids, has been reported to be downregulated in PTSD patients,[42] as well as in animals exposed to long term stress.[9] A recent study suggested TSPO situated in hippocampal dentate gyrus to be involved in modulation of PTSD symptoms in an animal model. Its hippocampal overexpression mediated anti-PTSD mechanism is partially dependent on upregulation of APG followed by incitement of adult hippocampal neuro-biogenesis.[22] TSPO ligands upon oral administration herald surge in APG synthesis in the brain and exerts favorable anti-PTSD effect.[43,44] Our data also showed that TSPO expression was significantly lowered in the CF^+/SPS^+ animals that resulted in simultaneous depletion in APG levels which were consistently increased upon treatment with TnCE and was comparable to that of SeR. Thus, the recovered TSPO levels by TnCE stimulated APG mediated GABAergic neurotransmission that promoted alleviation of the PTSD symptoms in CF^+/SPS^+ mice. These molecular alterations were evidently reflected in the histopathological observations as the TnCE treated hippocampal tissue retained their normal microarchitecture which was disrupted in the PTSD-inflicted mice. Interestingly, we also found that pre-treatment with PK11195 prior to TnCE markedly inhibited the overexpression of TSPO protein, which, in turn, negatively impacted APG biogenesis and GABA neurotransmission, thus validating the attenuation of all the positive behavioral responses induced by TnCE as observed in the behavioral tests. Such results correlated with previous experiments which demonstrated the activities of TSPO ligands to be completely abolished by PK11195.[45]

CONCLUSION

With the multi-faceted efficacy of TnCE, the current study revealed that repeated administration of the plant extract could reverse PTSD-induced anxiogenic and depressive behaviors along with extinction of contextual memory. Even though it cannot be confirmed completely, the current study verified that the molecular mechanism underlying the anti-PTSD potency of TnCE is associated with TSPO upregulation and is partly facilitated by APG biogenesis mediated neurotransmission. Thus, TnCE may be highlighted as a promising new pharmaco-therapeutic method for future PTSD treatment.

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Conflicts of interest

There are no conflicts of interest.

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