Molecular Investigation of Fluoxetine and *Fagonia indica*Combination Therapy for Stress-Induced Cognitive Impairment

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Abstract

Background

Chronic stress is a global health crisis that precipitates structural and functional deterioration of key cognitive brain regions, including the hippocampus and prefrontal cortex, leading to downregulation of critical neurotrophic and cholinergic markers. Fluoxetine, a widely prescribed selective serotonin reuptake inhibitor (SSRI), carries a burden of adverse effects with long-term use, while *Fagonia indica*, a phytomedicinal herb of the Zygophyllaceae family with established antioxidant, anti-inflammatory, and neuroprotective properties, remains unexplored in the context of cognitive impairment.

Objective

To investigate the molecular effects of Fluoxetine and *Fagonia indica*, individually and in combination, on stress-induced alterations in brain-derived neurotrophic factor (BDNF) and muscarinic acetylcholine receptor M3 (M3) expression in the cortex and hippocampus of chronically stressed female BALB/c mice.

Methods

Eighty non-pregnant female BALB/c mice (aged 8–12 weeks) were randomised into eight groups (n = 10). Chronic restraint stress was induced for 4 hours daily over 30 days. Treatments administered orally included Fluoxetine (18 mg/kg/day) and *Fagonia indica* methanolic extract (400 mg/kg/day), both individually and in combination. Brain cortex and hippocampus were dissected, and RNA was extracted using TRIzol reagent. Quantitative RT-PCR was performed using SYBR Green chemistry with β-actin as a housekeeping gene. Relative gene expression was calculated by the 2^-ΔΔCt method. Statistical analysis employed one-way ANOVA with Tukey's post hoc test ($p \leq 0.05$).

Results

No statistically significant differences in body weight were observed across groups. In the cortex, BDNF expression was reduced in the stress group (0.80 ± 0.19), with further suppression in Fluoxetine (0.72 ± 0.05) and *Fagonia indica* monotherapy (0.55 ± 0.04) groups. The combination therapy (St + Flx+Fag) yielded the highest cortical BDNF restoration (1.06 ± 0.09). In the hippocampus, combination therapy achieved a 2.14 ± 0.41-fold increase in BDNF expression, significantly superior to both monotherapies ($p < 0.05$) and the stress group ($p < 0.01$). Muscarinic M3 expression followed a similar pattern: combination therapy produced the highest expression in both brain regions (cortex: 1.45 ± 0.34; hippocampus: 2.56 ± 1.72), though hippocampal M3 changes did not reach statistical significance due to high intra-group variability.

Conclusion

Significant cognitive function restoration was observed by *Fagonia indica* by improving both BDNF and M3 expression in the combination therapy groups. These findings highlight the neuroprotective and cognitive potential of *Fagonia indica* for combination therapy for chronic stress management as a natural derivative. Further analysis of its pharmacokinetic studies is needed to develop its long-term safety profile.

1. INTRODUCTION

Stress-related disorders represent one of the most pervasive public health challenges of the twenty-first century. A 2024 Ipsos global survey spanning 31 countries found that 62% of respondents experienced stress severe enough to impair daily functioning, with a markedly higher prevalence in women (66%) compared to men (58%), and disproportionate burden in younger populations (Ipsos, 2025). A large-scale meta-analysis encompassing 149 countries and the period 2007–2021 documented a significant deterioration in population-level psychological stress, with more than half of the populations across 20 countries reporting emotional distress, and 85% of countries showing worsened stress profiles by 2020 relative to 2008 (Piao et al., 2024). These epidemiological trends underscore an urgent need for improved therapeutic strategies that go beyond current pharmacological paradigms.

At the neurobiological level, chronic stress disrupts homeostatic equilibrium through coordinated activation of the hypothalamic pituitary adrenal (HPA) axis and the sympathetic–adrenal–medullary (SAM) axis. Sustained elevation of glucocorticoids principally cortisol in humans and corticosterone in rodents induces neuroinflammation, oxidative stress, and excitotoxic glutamate release, culminating in structural remodelling of stress-sensitive brain regions (García-Bueno et al., 2008; Ulrich-Lai & Herman, 2009). The hippocampus, a structure essential for the encoding and retrieval of declarative memory, and the prefrontal cortex, which governs executive function and attentional regulation, are particularly susceptible to glucocorticoid-mediated toxicity (McEwen & Morrison, 2013). Chronic stress reduces dendritic complexity, impairs neurogenesis, and suppresses synaptic plasticity in these regions, thereby producing deficits in learning, working memory, and cognitive flexibility (Schoenfeld et al., 2017).

Among the molecular mediators of stress-related cognitive impairment, brain-derived neurotrophic factor (BDNF) occupies a central role. BDNF is an activity-dependent neurotrophin that sustains synaptic integrity through TrkB receptor–mediated activation of the MAPK/ERK and PI3K/Akt pathways, promoting neuronal survival, long-term potentiation, and dendritic arborisation (Bath et al., 2013). Chronic stress consistently downregulates BDNF expression in the hippocampus and prefrontal cortex, and this suppression correlates mechanistically with impaired synaptic signalling and accelerated neuronal atrophy (Morrison & Baxter, 2012; Algaidi, 2025). The neurotrophic hypothesis of cognitive impairment thus positions BDNF restoration as a principal therapeutic target.

Cholinergic signalling constitutes an equally important determinant of cognitive performance. The muscarinic acetylcholine receptor subtype M3 (M3), a Gq-coupled G protein–coupled receptor expressed throughout the hippocampus and cortex, participates in the modulation of attention, working memory consolidation, and cortical arousal (Smith et al., 2024). Chronic stress is associated with dysregulation of muscarinic receptor expression, and disruption of M3-mediated cholinergic neurotransmission has been implicated in the cognitive sequelae of stress-related neuropsychiatric disorders (Khalid et al., 2017). Despite its functional importance, M3 receptor expression in the context of pharmacological stress management remains inadequately characterised.

The current pharmacological standard for stress-related disorders is dominated by selective serotonin reuptake inhibitors (SSRIs). Fluoxetine, the prototypical SSRI marketed as Prozac, inhibits the presynaptic serotonin transporter (SERT) and increases synaptic serotonin bioavailability, thereby enhancing mood, emotional resilience, and anxiety tolerance (Edinoff et al., 2021). Fluoxetine also exerts BDNF-upregulatory effects through serotonin-dependent stimulation of TrkB signalling and hippocampal neurogenesis (David et al., 2009). However, its clinical utility is constrained by a well-documented adverse effect profile including nausea, sexual dysfunction, insomnia, and with prolonged use emotional blunting, cognitive decline, and increased haemorrhagic risk (Marazziti et al., 2019; Robert & Hirschfeld, 2003). Concerns about dependency and withdrawal further limit its long-term tolerability.

These limitations have catalysed a paradigmatic shift towards complementary and alternative medicine (CAM), and specifically towards bioactive phytochemicals capable of modulating multiple neuropathological pathways simultaneously. *Fagonia indica* (family Zygophyllaceae), locally known in Pakistan as Dhamasa or Suchi Booti, is a phytomedicinal herb with a rich ethnopharmacological tradition in South Asia and the Middle East. Its phytochemical constituents including flavonoids, saponins, polyphenols, and alkaloids confer potent antioxidant, anti-inflammatory, immunomodulatory, hepatoprotective, and gastroprotective activities (Ali & Khan, 2021; Almilaibary et al., 2022). Related species such as *Fagonia arabica* have demonstrated neuroprotection in PC12 cells under chemical ischaemia (Satpute et al., 2012), while *Fagonia cretica* has been shown to modulate hippocampal antioxidant defences under oxygen glucose deprivation. Nevertheless, the direct molecular effects of *Fagonia indica* on stress-induced cognitive biomarkers remain unexplored.

The concept of combination therapy pairing a conventional psychopharmacological agent with a phytochemical adjunct presents a compelling strategy to achieve synergistic neuroprotection while potentially reducing the dose-dependent adverse effects associated with monotherapy. Such integrative approaches have demonstrated additive or synergistic effects in preclinical models of neurodegeneration and cognitive impairment, yet no study has examined the joint molecular effects of Fluoxetine and *Fagonia indica* on BDNF and M3 receptor expression under chronic stress conditions.

The present study was therefore designed to address this gap by quantifying the mRNA expression of BDNF and the M3 muscarinic receptor in the cortex and hippocampus of chronically stressed female BALB/c mice treated with Fluoxetine, *Fagonia indica*, or their combination. We hypothesised that

combination therapy would elicit superior restoration of both cognitive markers compared to either monotherapy, reflecting a synergistic neuroprotective and cholinergic-restorative mechanism. The specific objectives were: (1) to establish a validated murine restraint stress model of cognitive impairment; (2) to quantify region-specific changes in BDNF and M3 expression across treatment groups using RT-PCR; and (3) to evaluate the comparative efficacy of monotherapy versus combination therapy in restoring molecular correlates of cognitive function.

2. LITERATURE REVIEW

2.1 Stress, the HPA Axis, and Cognitive Impairment

Stress is defined as any condition in which internal or external stimuli challenge an organism's physiological homeostasis (Tafet & Bernardini, 2003). The stress response is orchestrated by the HPA axis and the sympathetic nervous system (SNS). Environmental or psychological stressors generate cortical neuronal activity that propagates to the limbic system, triggering hypothalamic secretion of corticotrophin-releasing factor (CRF). CRF stimulates anterior pituitary release of adrenocorticotrophic hormone (ACTH), which acts on the adrenal cortex to produce glucocorticoids—cortisol in humans and corticosterone in rodents (Ulrich-Lai & Herman, 2009). Parallel activation of the SNS produces catecholamines (adrenaline, noradrenaline) that amplify the physiological stress response.

Acute stress exerts an adaptive, memory-facilitating effect mediated by moderate glucocorticoid receptor (GR) activation that enhances synaptic consolidation (Lindau et al., 2016). However, chronic stress induces glucocorticoid resistance a state in which immune cells become desensitised to the anti-inflammatory actions of cortisol resulting in unchecked neuroinflammation characterised by elevated pro-inflammatory cytokines (IL-1β, TNF-α, IL-6) and reactive oxygen species (ROS) (Zefferino et al., 2021). This neuroinflammatory milieu, combined with excitotoxic glutamate overflow, drives dendritic retraction, synaptic pruning, and suppression of adult hippocampal neurogenesis, collectively impairing cognitive performance (McEwen & Morrison, 2013; Schoenfeld et al., 2017).

The hippocampus, richly endowed with both mineralocorticoid receptors (MR) and glucocorticoid receptors (GR), is the brain region most vulnerable to glucocorticoid neurotoxicity. Structural magnetic resonance imaging studies in humans consistently demonstrate hippocampal volume reduction in individuals with chronic stress disorders, post-traumatic stress disorder (PTSD), and major depressive disorder (MDD), findings that are recapitulated in rodent restraint stress models (Zain et al., 2019; Alqurashi et al., 2022). The prefrontal cortex similarly undergoes dendritic atrophy and impaired long-term potentiation under chronic stress, compromising executive function, working memory maintenance, and attentional control (Algaidi, 2025).

2.2 BDNF Signalling and Stress-Induced Neuroplasticity Deficits

BDNF is a member of the neurotrophin family that signals primarily through the high-affinity tyrosine kinase receptor TrkB. Upon BDNF binding, TrkB undergoes autophosphorylation and activates three canonical downstream pathways: (1) the MAPK/ERK cascade, which promotes neuronal differentiation and long-term synaptic potentiation; (2) the PI3K/Akt pathway, which enhances neuronal survival and suppresses apoptosis; and (3) phospholipase Cγ1 (PLCγ1), which regulates short-term synaptic plasticity through Ca^{2+}-dependent mechanisms (Bath et al., 2013). These intracellular signalling cascades collectively sustain dendritic complexity, spine density, and the molecular substrates of long-term memory formation.

Chronic stress robustly downregulates BDNF mRNA and protein expression in the hippocampus and prefrontal cortex, mediated in part by glucocorticoid-induced epigenetic modifications including histone deacetylation and increased promoter methylation of the BDNF gene (Morrison & Baxter, 2012). Reduced BDNF-TrkB signalling impairs synaptic plasticity and reduces dendritic arborisation, creating a molecular substrate for the learning and memory deficits associated with chronic stress. Conversely, restoration of BDNF expression through pharmacological or phytochemical intervention has been correlated with improved cognitive performance in multiple rodent models, positioning BDNF upregulation as a primary therapeutic endpoint (Algaidi, 2025).

2.3 Muscarinic M3 Receptor and Cholinergic Modulation of Cognition

The cholinergic system, comprising acetylcholine (ACh)-producing basal forebrain neurons and their cortical and hippocampal projections, is a principal mediator of attentional processing, working memory, and mnemonic consolidation. Five muscarinic receptor subtypes (M1–M5) and nicotinic receptors mediate the central effects of ACh. The M3 receptor, a Gq/11-coupled subtype, is broadly expressed across the hippocampus and cortex and participates in the modulation of cortical arousal, attentional gating, and hippocampal-dependent memory (Smith et al., 2024). Immobilisation stress has been shown to increase brain acetylcholinesterase (AChE) activity, accelerating ACh degradation and impairing cholinergic neurotransmission in a fashion that correlates with cognitive deficits (Das et al., 2000). Altered muscarinic receptor expression under stress conditions—particularly in the hippocampus may further compromise cholinergic efficacy by reducing receptor-mediated signal transduction (Khalid et al., 2017).

2.4 Fluoxetine: Pharmacology and Neuroprotective Mechanisms

Fluoxetine (Prozac) is the oldest SSRI, inhibiting SERT to increase synaptic serotonin concentration. It undergoes hepatic metabolism via cytochrome P450 2D6 (CYP2D6) to its active metabolite norfluoxetine, which has a substantially extended half-life, affording a gradual pharmacological offset that mitigates abrupt discontinuation syndrome (Edinoff et al., 2021). Beyond serotoninergic activity, fluoxetine exerts neuroprotective effects through BDNF-TrkB upregulation, enhancement of hippocampal neurogenesis, and anti-inflammatory modulation of microglial activation via downregulation of pro-

inflammatory cytokine production (Tynan et al., 2012). Chronic fluoxetine administration in BALB/c mice at 18 mg/kg/day has been shown to produce anxiolytic and antidepressant effects and to significantly reduce immobility in the forced swim test, validating this dosage for preclinical cognitive studies (Dulawa et al., 2004a; Holick et al., 2008).

Despite these beneficial neurobiological effects, long-term fluoxetine use is associated with cognitive side effects including emotional numbing and impaired processing speed, as well as systemic adverse events such as sexual dysfunction, gastrointestinal disturbance, and increased osteoporotic fracture risk (Marazziti et al., 2019; Cipriani et al., 2009). These limitations motivate the exploration of adjunctive strategies that can amplify fluoxetine's neuroprotective properties while attenuating its adverse effect burden.

2.5 *Fagonia indica*: Phytochemistry and Neuroprotective Potential

Fagonia indica is a spiny perennial herb of the Zygophyllaceae family distributed across arid and semi-arid regions of South Asia, the Middle East, North Africa, and the Mediterranean basin.(Fig. 1) In Pakistan, it is encountered throughout dry terrains and is known by the vernacular names Dhamasa and Suchi Booti (Ali & Khan, 2021). Phytochemical analyses have identified a complex array of bioactive constituents, including saponins, flavonoids (quercetin, kaempferol), polyphenols, alkaloids, and terpenoids. Pharmacologically, *Fagonia indica* exhibits antioxidant, anti-inflammatory, antimicrobial, immunomodulatory, anticoagulant, hepatoprotective, nephroprotective, and gastroprotective activities (Atiq-ur-Rehman et al., 2021).

The antioxidant capacity of *Fagonia indica*, attributable largely to its flavonoid and polyphenol content, is mechanistically relevant to neuroprotection: oxidative stress is a key driver of stress-induced neuronal damage, and compounds capable of quenching ROS can attenuate lipid peroxidation, protein carbonylation, and DNA oxidative strand breaks in vulnerable neurons. Related species lend support to this neuroprotective potential: *Fagonia arabica* preserved neuronal viability in PC12 cells under chemical ischaemia by maintaining antioxidant enzyme activity (Satpute et al., 2012), and *Fagonia olivieri* administered at 400 mg/kg/day demonstrated hepatoprotective and metabolic regulatory effects in rodents (Rashid et al., 2016). Direct molecular evidence for *Fagonia indica* 's effects on BDNF or muscarinic receptor expression in stress-exposed brain tissue has not been previously reported, constituting the principal knowledge gap addressed by the present investigation.

2.6 Combination Therapy and Research Gaps

The integration of phytomedicinal agents with conventional psychopharmacological drugs represents a rational therapeutic strategy grounded in the principle of multi-target pharmacology. Herbal compounds capable of modulating oxidative stress, neuroinflammation, and neurotrophic signalling may complement SSRIs that primarily act through serotoninergic mechanisms, creating additive or synergistic neuroprotective outcomes. Several preclinical studies have demonstrated enhanced

cognitive restoration through phytochemical–drug combinations compared to monotherapy in rodent models of neurodegeneration. Turmeric (curcumin) combined with conventional agents improved muscarinic receptor expression in stress models (Khalid et al., 2017). However, the specific combination of fluoxetine and *Fagonia indica*, and its molecular impact on BDNF and M3 receptor expression, has not been investigated.

3. MATERIALS AND METHODS

3.1 Ethical Approval

All animal experimental procedures received prior approval from the Institutional Review Board (IRB) of the National University of Sciences and Technology (NUST), Pakistan (IRB No. 11-2022-ASAB-01/03). The study was conducted in strict accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals (8th Edition, 2011) and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines to ensure rigorous and transparent reporting.

3.2 Animals and Housing Conditions

Eighty non-pregnant female BALB/c mice, aged 8–12 weeks with a body weight of 25–35 g, were procured from the National Institute of Health, Islamabad, Pakistan. Animals were housed in the Animal House Laboratory, NUST, under controlled environmental conditions (22 ± 2°C, 14:10-hour light/dark cycle). Six to eight animals were housed per polypropylene cage (40 cm × 25 cm × 15 cm) with sterile wood-chip bedding, with free access to standard rodent chow and distilled water ad libitum. All animals underwent a one-week acclimatisation period prior to experimental procedures. Animals with visible signs of illness, pregnancy, or abnormal body weight were excluded.

3.3 Plant Material and Extract Preparation

Dried aerial parts of *Fagonia indica* were purchased from a certified local herbal pharmacy (pansaar) in Islamabad and authenticated by a qualified plant taxonomist. The methanolic extract was prepared by the maceration technique. Briefly, the dried plant material was ground to a fine powder for 60 seconds using an electric grinder. Ten grams of powdered material was placed in amber-coloured borosilicate glass bottles and macerated in 200 mL of analytical-grade methanol (1:20 w/v) for 10 days in a dark cabinet with periodic agitation. The extract was filtered through Whatman No. 42 filter paper, and the filtrate was transferred to Petri plates for solvent evaporation at 37°C in an incubator for 24 hours. The dried extract was stored at 4°C until use. (Figure 2)

3.4 Experimental Design and Treatment Groups

The study was designed as a parallel-group, randomised controlled preclinical experiment of 30 days duration. Eighty mice were randomly allocated to eight groups (n = 10 per group) as follows:

- Group 1 – Control (C): Standard housing conditions; no stress induction; no pharmacological intervention.
- Group 2 – Stress (St): Daily 4-hour restraint stress for 30 days; no treatment.
- Group 3 – Fluoxetine (Flx): Fluoxetine 18 mg/kg/day orally; no stress.
- Group 4 – *Fagonia indica* (Fag): *Fagonia indica* extract 400 mg/kg/day orally; no stress.
- Group 5 – Fluoxetine + *Fagonia indica*(Flx+Fag): Fluoxetine 18 mg/kg/day + *Fagonia indica* 400 mg/kg/day orally; no stress.
- Group 6 – Stress + Fluoxetine (St+Flx): Daily restraint stress + Fluoxetine 18 mg/kg/day orally.
- Group 7 – Stress + *Fagonia indica*(St+Fag): Daily restraint stress + *Fagonia indica* 400 mg/kg/day orally.
- Group 8 – Stress + Fluoxetine + *Fagonia indica* (St+Flx+Fag): Daily restraint stress + Fluoxetine 18 mg/kg/day + *Fagonia indica* 400 mg/kg/day orally.

3.5 Restraint Stress Protocol

Chronic restraint stress was induced using a modified immobilisation protocol (Khalid et al., 2017). Between 09:00 and 13:00 hours daily, mice in all stressed groups were individually confined in body-fitting cylindrical restrainers fabricated from 50 mL polypropylene Falcon tubes. A 5 mm ventilation aperture was made at the distal end and a tail-exit port at the proximal end, preventing locomotion while permitting unrestricted respiration. Mice were restrained for 4 consecutive hours per day for 30 days. Control and drug-only groups were not subjected to any stress procedure.

3.6 Drug Preparation and Oral Administration

Fluoxetine hydrochloride (Prozac 20 mg capsules; Eli Lilly Pakistan Pvt. Ltd.) was dissolved and incorporated into finely powdered standard rodent chow to achieve a dose of 18 mg/kg/day, validated in prior studies as the most effective chronic dosage for BALB/c mice (Dulawa et al., 2004a; Holick et al., 2008). *Fagonia indica* methanolic extract was similarly admixed to achieve 400 mg/kg/day, based on an effective dose established for the closely related species *Fagonia olivieri* in rodents (Rashid et al., 2016). Dosing calculations were based on pilot study data demonstrating average daily feed consumption of approximately 7 g in a 32 g mouse; accordingly, 8.3 mg Fluoxetine and 142.8 mg *Fagonia indica* extract were added per 100 g of crushed feed, formed into pellets with distilled water, and air-dried. Medicated pellets were provided as the sole feed source throughout the 30-day treatment period.

3.7 Animal Sacrifice and Tissue Collection

On day 30, all animals were sacrificed at approximately the same time of day (14:00–15:00 hours) to control for circadian variation in glucocorticoid and neurotrophic factor levels. Animals were deeply anaesthetised with chloroform (~1 mL in a closed chamber), followed by rapid decapitation using a sterile surgical kit. The cranium was opened, the brain was carefully excised, and the cortex and hippocampus were rapidly microdissected on a sterile ice-cold surface and snap-frozen at −80°C until

molecular analysis. Blood was collected via cardiac puncture into ACD (Acid Citrate Dextrose) anticoagulant tubes, centrifuged at 13,000 rpm for 10 minutes to isolate serum, which was stored at −20°C.

3.8 RNA Extraction

Total RNA was extracted from cortex and hippocampus tissue using TRIzol Reagent (bioZOL™-G; Bioworld; Cat. No. 10760055-1) following the manufacturer's protocol with modifications. Briefly, tissue samples were homogenised in 1 mL TRIzol using an ultrasonic processor (Hielscher UP400S) and incubated at 4°C for 15 minutes. Chloroform (200 µL) was added, and samples were vortexed for 15 seconds and incubated at 4°C for 10 minutes before centrifugation at 12,000 rpm for 15 minutes at 4°C. The upper aqueous phase containing RNA was transferred to new autoclaved microcentrifuge tubes. RNA was precipitated with 500 µL isopropanol (10 minutes, room temperature), pelleted at 12,000 rpm for 10 minutes, washed with 1 mL absolute ethanol, and centrifuged at 7,500 × g for 5 minutes at 4°C. After air-drying, the pellet was resuspended in 30–40 µL nuclease-free water (Invitrogen; Cat. No. AM9932) and stored at −80°C.

3.9 RNA Quantification and Quality Assessment

RNA concentration and purity were determined using a Thermo Scientific NanoDrop ND-2000 spectrophotometer. Each sample was measured in triplicate. Samples were considered acceptable for downstream applications when the A260/280 ratio was approximately 2.0 (indicating minimal protein contamination) and concentration fell within the 200–500 ng/µL range.

3.10 cDNA Synthesis

Complementary DNA (cDNA) was synthesised from 2 µg of total RNA per sample using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Cat. No. K1622) according to the manufacturer's instructions. cDNA integrity was verified by conventional PCR amplification of the housekeeping gene β-actin (Mus musculus; Forward: 5'-GGCTGTATTCCCCTCCATCG-3'; Reverse: 5'-CCAGTTGGTAACAATGCCATGT-3'), yielding a 359 bp amplicon visualised on a 2% agarose gel under UV transillumination. Synthesised cDNA was stored at −20°C.

3.11 Quantitative RT-PCR

Quantitative real-time PCR was performed using WizPure™ qPCR Master Mix (SYBR Green; Cat. No. W1711) on a Mic qPCR Cycler (Bio Molecular Systems). Each 10 µL reaction contained: 3.6 µL nuclease-free water, 0.2 µL each of forward and reverse primer (10 µM stock), 4 µL SYBR Green Master Mix, and 2 µL cDNA template. The thermocycling programme comprised an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 10 seconds (denaturation), 53°C for BDNF / 55°C for M3 for 30 seconds (annealing), and 72°C for 10 seconds (extension), with a final extension at 72°C for 5 minutes. Melt-curve analysis was performed to confirm amplicon specificity. Gene-specific primer sequences are provided in Table 1.

3.12 Gene Expression Analysis

Relative mRNA expression was calculated using the comparative 2^-ΔΔCt method. The cycle threshold (Ct) of each target gene was normalised to β-actin (ΔCt = Ct_target − Ct_β-actin). ΔΔCt was calculated as ΔCt_treatment group − ΔCt_control group, and relative fold-change was expressed as 2^-ΔΔCt. The control group was set to a relative expression of 1. Each PCR reaction was performed in triplicate (n = 3 biological replicates per group), and values deviating beyond two standard errors of the mean (SEM) were treated as outliers and excluded.

3.13 Statistical Analysis

All data are expressed as mean ± SEM (n = 3). Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons post hoc test in GraphPad Prism version 8 (GraphPad Software, Inc., San Diego, CA, USA). A p-value of ≤ 0.05 was considered statistically significant. Significance thresholds are denoted as: *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

4. RESULTS

4.1 RNA and cDNA Quality Control

Total RNA was successfully extracted from cortex and hippocampus samples across all experimental groups. Spectrophotometric analysis using the NanoDrop ND-2000 yielded A260/280 ratios ranging from 1.49 to 2.11, with the majority of samples falling within the acceptable purity range of 1.8–2.0 (Table 2,3). Similarly, synthesised cDNA samples demonstrated A260/280 ratios of 1.59–1.97, confirming adequate purity for downstream quantitative PCR. cDNA integrity was further verified by amplification of a 359 bp β-actin amplicon on a 2% agarose gel, with consistent band intensity observed across all samples (Figure 3).

4.2 Body Weight Analysis

No statistically significant intra-group variation in body weight was observed between the beginning and end of the 30-day experimental period within any individual group. Body weight remained stable across all treated and stressed groups throughout the study, indicating adequate nutritional intake and ruling out significant systemic toxicity from either treatment. Inter-group comparison on Day 30 revealed that the control group exhibited the greatest cumulative weight gain, resulting in a statistically significant weight differential relative to stress-exposed and treatment-receiving groups. This is consistent with previous reports of stress-induced metabolic disturbances and SSRI-related appetite modulation rather than active weight loss in treated groups (Loprinzi & Frith, 2019; Lyons et al., 2012).

4.3 BDNF Expression Analysis

4.3.1 BDNF Expression in the Cerebral Cortex

Relative BDNF mRNA expression in the cerebral cortex is presented in Figure 2. The stress group exhibited a non-significant reduction in cortical BDNF expression (0.80 ± 0.19; ns) relative to the control group (set as 1.00). Among stress-exposed treatment groups, both Fluoxetine monotherapy (St+Flx: 0.72 ± 0.05; ns) and *Fagonia indica* monotherapy (St+Fag: 0.55 ± 0.04; ns) produced further decreases in cortical BDNF levels, suggesting insufficient neuroprotective effect at the cortical level when administered alone. In stark contrast, the combination therapy group (St+Flx+Fag) achieved the highest cortical BDNF expression across all stress-treated groups (1.06 ± 0.09; *p ≤ 0.05 versus *Fagonia indica* monotherapy), demonstrating near-complete restoration to control levels and indicating a synergistic BDNF-upregulatory effect. In non-stressed treated groups (Flx, Fag, Flx+Fag), no significant changes in cortical BDNF expression were observed relative to control. (Graph 1)

4.3.2 BDNF Expression in the Hippocampus

Hippocampal BDNF expression data are presented in Figure 3. The stress group demonstrated a pronounced though non-statistically-significant reduction in hippocampal BDNF (0.32 ± 0.02; ns versus control), reflecting the heightened vulnerability of the hippocampus to glucocorticoid-mediated BDNF suppression. Both Fluoxetine monotherapy (St+Flx: 0.47 ± 0.09; ns) and *Fagonia indica* monotherapy (St+Fag: 0.41 ± 0.09; ns) failed to produce significant recovery of hippocampal BDNF, remaining substantially below control values. The combination therapy group (St+Flx+Fag) achieved remarkable hippocampal BDNF upregulation (2.14 ± 0.41), representing a 6.7-fold increase relative to the stress group. This restoration was statistically significant versus the stress group (**p < 0.01), versus Fluoxetine monotherapy (*p < 0.05), and versus *Fagonia indica* monotherapy (*p < 0.05), confirming genuine synergism between the two therapeutic agents in hippocampal BDNF restoration. (Graph 2)

4.4 Muscarinic M3 Receptor Expression Analysis

4.4.1 M3 Expression in the Cerebral Cortex

Cortical M3 receptor mRNA expression across treatment groups is illustrated in Figure 4. The stress group exhibited a non-significant downregulation of M3 (0.76 ± 0.32; ns) relative to control, indicating modest cortical cholinergic disruption under chronic restraint stress. Fluoxetine monotherapy (St+Flx: 0.56 ± 0.02; ns) produced further M3 suppression, suggesting that fluoxetine alone does not favourably modulate cortical cholinergic receptor expression. *Fagonia indica* monotherapy (St+Fag: 0.93 ± 0.70; ns) achieved near-restoration of M3 expression to control values. The combination therapy group (St+Flx+Fag) demonstrated the highest cortical M3 expression (1.45 ± 0.34; ns), exceeding control levels, although the high intra-group variability precluded statistical significance. In non-stressed groups, no significant treatment-related changes in M3 expression were observed. (Graph 3)

4.4.2 M3 Expression in the Hippocampus

Hippocampal M3 receptor expression is shown in Figure 5. The stress group displayed a marked reduction in hippocampal M3 expression (0.33 ± 0.02; ns versus control), reflecting significant disruption

of hippocampal cholinergic signalling by chronic stress. Fluoxetine monotherapy (St+Flx: 0.59 ± 0.03; ns) and *Fagonia indica* monotherapy (St+Fag: 0.82 ± 0.22; ns) both resulted in partial, non-significant improvements. The combination therapy group (St+Flx+Fag) produced the highest hippocampal M3 expression (2.56 ± 1.72; ns), representing a 7.8-fold increase relative to the stress group, although the large SEM attributable to biological variability prevented statistical significance. This trend nonetheless strongly suggests an additive or synergistic cholinergic restorative effect. In non-stressed treatment groups, M3 expression remained comparable to control values.(Graph 4)

5. DISCUSSION

This study provides the first molecular evidence demonstrating that co-administration of Fluoxetine and *Fagonia indica* methanolic extract produces superior restoration of hippocampal BDNF expression and the highest M3 muscarinic receptor upregulation in both cortex and hippocampus compared to either monotherapy in a murine model of chronic restraint stress. These findings carry significant implications for the development of integrative neuroprotective strategies targeting stress-induced cognitive impairment.

The chronic restraint stress paradigm employed in this study is a well-validated model that simultaneously engages physical and psychological stress pathways, replicating aspects of real-world chronic stress exposure (Radley & Morrison, 2005). Restraint stress activates the HPA axis, elevates corticosterone, and produces structural brain modifications including dendritic retraction, impaired neurogenesis, and reduced synaptic density in the hippocampus and prefrontal cortex (Das et al., 2000). The absence of significant body weight loss in stress-exposed groups, as observed in the present study, is consistent with a growing body of evidence suggesting that weight effects in restraint stress models are highly variable and dependent on stress intensity, duration, and animal sex (Martí et al., 1994; Loprinzi & Frith, 2019).

The observed suppression of cortical and hippocampal BDNF in the stress group is consistent with the neurotrophic hypothesis of stress-related cognitive dysfunction. Glucocorticoid excess represses BDNF gene expression through GR-mediated transcriptional suppression and epigenetic modifications, producing a state of neurotrophic deficit that manifests as impaired synaptic plasticity and neuronal atrophy (Morrison & Baxter, 2012; Algaidi, 2025). The differential magnitude of BDNF suppression between cortex and hippocampus observed here with hippocampal suppression reaching 68% of control versus 20% in cortex corroborates region-specific BDNF regulatory mechanisms. The hippocampus is enriched with GRs and represents the primary site of glucocorticoid-mediated BDNF suppression, whereas the cortex may be partially buffered by compensatory neurotrophic mechanisms (Esvald et al., 2022).

The failure of Fluoxetine monotherapy to significantly restore cortical BDNF expression, and its limited hippocampal effect, was unexpected given the established BDNF-upregulatory capacity of SSRIs through serotonin-TrkB signalling (David et al., 2009). This may reflect dose- or time-dependent limitations of

fluoxetine's neurotrophic effects under conditions of concurrent severe chronic stress, where sustained glucocorticoid excess may counteract fluoxetine-induced BDNF transcription. Similar dissociations between SSRI administration and BDNF restoration have been reported in other stress models, particularly with shorter treatment durations (Erburu et al., 2015). Additionally, the stress-induced suppression of neurogenesis may reduce the neurogenic substrate through which fluoxetine's BDNF effects are typically expressed (Schoenfeld et al., 2017).

The neuroprotective effects of *Fagonia indica* are mechanistically attributable to its phytochemical constituents. Flavonoids such as quercetin and kaempferol are potent radical scavengers that inhibit lipid peroxidation and attenuate ROS-mediated suppression of BDNF promoter activity, while saponins and polyphenols modulate the NF-κB inflammatory pathway to reduce neuroinflammation (Ali & Khan, 2021). By reducing oxidative-neuroinflammatory stress a principal upstream driver of BDNF suppression *Fagonia indica* may preserve the transcriptional machinery necessary for BDNF gene expression. These mechanisms are supported by the demonstrated antioxidant and neuroprotective activities of related Fagonia species in neuronal injury models (Satpute et al., 2012).

The remarkable synergy observed in hippocampal BDNF expression under combination therapy (2.14-fold versus stressed monotherapies at 0.41–0.47-fold) suggests mechanistic complementarity between Fluoxetine and *Fagonia indica*. We propose that Fluoxetine provides serotoninergic stimulation of TrkB signalling and transcription factor activation (e.g., CREB phosphorylation), while *Fagonia indica* 's antioxidant and anti-inflammatory properties neutralise the oxidative-stress barrier that otherwise prevents Fluoxetine from fully upregulating BDNF under high glucocorticoid conditions. This multi-modal co-operation may explain why combination therapy achieved BDNF levels exceeding control values in the hippocampus a phenomenon of BDNF overshoot that may reflect compensatory neuroplastic responses when both transcriptional activation and transcriptional barriers are simultaneously addressed.

The pattern of M3 muscarinic receptor expression paralleled BDNF findings. Chronic stress reduced M3 expression in both regions, consistent with stress-induced acetylcholinesterase upregulation that disrupts cholinergic homeostasis and may downregulate receptor expression through receptor desensitisation or reduced ACh availability (Das et al., 2000; Khalid et al., 2017). Fluoxetine monotherapy failed to restore and even slightly reduced cortical M3 expression, consistent with the limited direct cholinergic activity of SSRIs—fluoxetine's mechanism does not involve direct muscarinic receptor modulation. By contrast, *Fagonia indica* 's polyphenolic constituents may indirectly support cholinergic neurotransmission through AChE inhibition and reduced cholinergic neurotoxicity, as suggested by the near-control M3 restoration in the St+Fag group. The combination therapy's M3 expression exceeding control values in both brain regions suggests a potentiating interaction: fluoxetine's serotoninergic stabilisation of cortical and hippocampal circuits may create a more favorable environment for *Fagonia indica*'s cholinergic-restorative actions (Fernández de Sevilla et al., 2021).

The high intra-group variability observed in hippocampal M3 expression in the combination therapy group (2.56 ± 1.72) likely reflects genuine biological heterogeneity in individual cholinergic receptor

expression responsiveness to combination treatment—an observation that calls for larger sample sizes in future confirmatory studies. The small n per group (n = 3 for molecular analysis) represents a notable limitation, as does the use of a single stress paradigm (restraint stress) which, while validated and reproducible, may not fully recapitulate the diversity of human stress experiences.

From a translational perspective, these findings are highly significant. Cognitive impairment secondary to chronic stress and related affective disorders represents a major unmet clinical need. Current SSRI monotherapy is inadequate in fully restoring neurotrophic and cholinergic function, as demonstrated here and in clinical literature. The identification of *Fagonia indica* as an adjunctive agent capable of potentiating SSRI-induced BDNF upregulation and restoring cholinergic receptor expression opens a promising avenue for integrative neuropharmacology. The oral bioavailability and established safety profile of *Fagonia indica* in traditional medicine, combined with its multi-target phytochemical composition, position it as an attractive candidate for clinical translation, provided that rigorous pharmacokinetic characterisation and controlled clinical trials are conducted.

6. CONCLUSION

The present study demonstrates that chronic restraint stress significantly suppresses the mRNA expression of BDNF and the muscarinic M3 receptor in the cortex and hippocampus of female BALB/c mice, establishing a molecular basis for stress-induced cognitive impairment. Fluoxetine monotherapy provided limited neuroprotection at the doses and duration examined, while *Fagonia indica* monotherapy demonstrated modest but noteworthy cholinergic restorative effects. Most significantly, co-administration of Fluoxetine and *Fagonia indica* achieved the highest expression of both BDNF and M3 receptor in both brain regions, with statistically significant hippocampal BDNF restoration surpassing both monotherapies and the stress control. These results provide compelling molecular evidence for the synergistic neuroprotective and cholinergic-restorative potential of this combination therapy. *Fagonia indica* emerges as a promising natural adjunct to conventional SSRI therapy, warranting further pharmacokinetic profiling, dose-optimisation studies, and controlled clinical evaluation for the management of stress-induced cognitive impairment.

Limitations and Future Directions

Future investigations should address the following limitations: (1) expansion of the stress paradigm repertoire to include chronic unpredictable stress and social defeat stress models for greater translational validity; (2) use of larger biological replicates (n ≥ 8–10 for molecular endpoints); (3) comprehensive pharmacokinetic and safety profiling of *Fagonia indica* including oral bioavailability, Cmax, half-life, and organ toxicity assessment; (4) protein-level validation of BDNF and M3 expression by ELISA and Western blotting; (5) behavioural cognitive assessments (Morris Water Maze, Novel Object Recognition) to correlate molecular findings with functional outcomes; and (6) investigation of *Fagonia indica* 's phytochemical fractions to identify the principal neuroprotective constituents.

Declarations

Authorship Contribution

F.K. designed the study, carried out the research, analyzed the data, and wrote the first draft of the publication. A.T. helped with the literature review, data collecting, and manuscript editing. W.A. helped with research, findings interpretation, and manuscript revision. A.O. developed the methodology, performed statistical analysis, and critically revised the text. S.H.A. helped with overall study coordination, paper review, project management, and supervision. The final draft of the work was examined and approved by all authors.

Clinical Registration number

Nil

Clinical Trial

Nil

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Tables

Tables 1 to 3 are available in the Supplementary Files section.

Figures

Figure 1

Morphology of Fagonia indica (*Fagonia Indica - Flora of Qatar*, 2025)

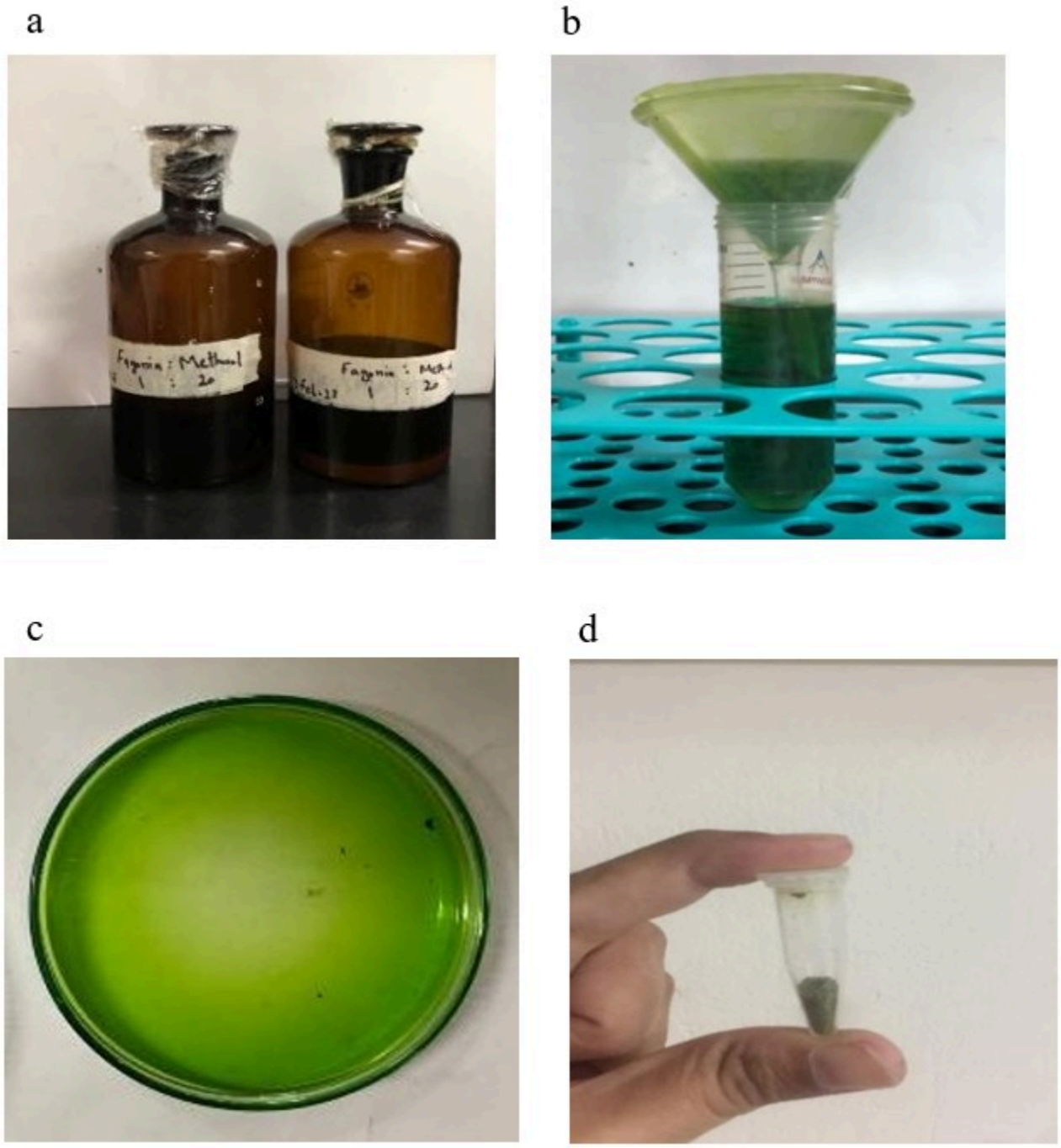

Figure 2

Plant extraction: (a) Maceration, (b) Filtration of extract, (c) Filtered extract poured in petri plates to be dried overnight at 37°C in incubator, (d) Dried extract to be stored at 4°C.

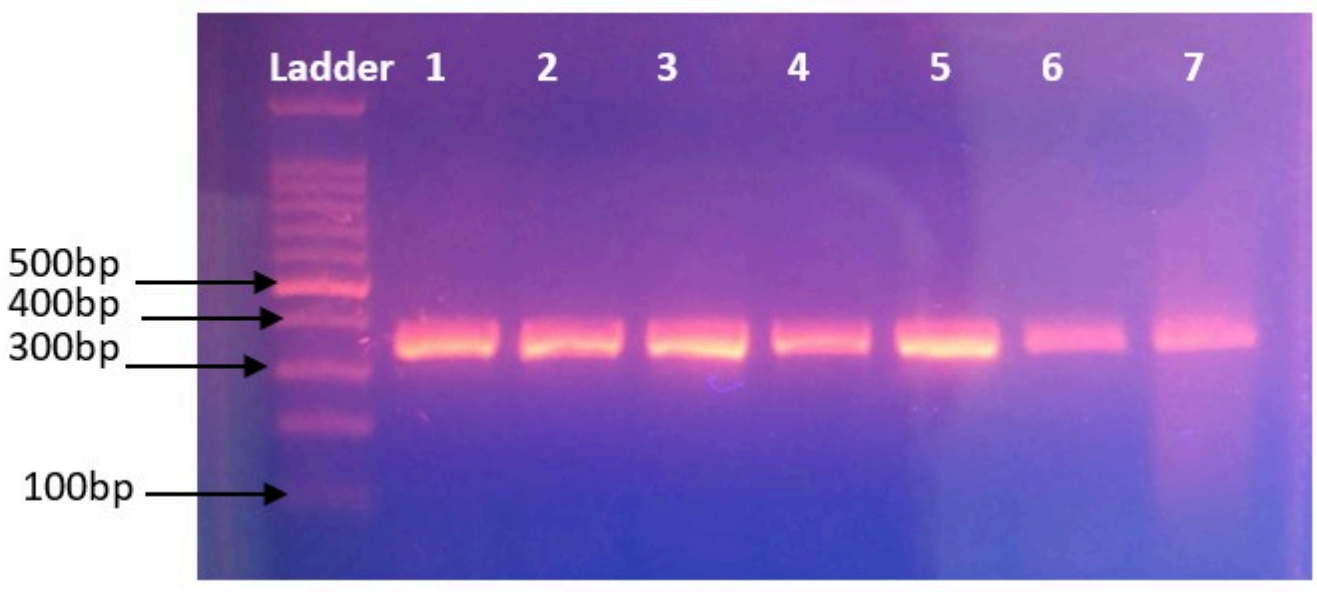

Figure 3

PCR-based amplification of cDNA synthesis confirmation. L represents the 1000bp ladder, 1-6 are cortex and hippocampus samples. 2% agarose gel was used.

Supplementary Files

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