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RESEARCH

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Neuroprotective effect of bromelain on BDNF-TRKB signalling pathway in chronic unpredictable stress-induced depression model

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Abstract

Background Bromelain is a mixture of protease enzyme extract from the fruit or stem of the pineapple plant. It has a wide range of biological actions, and it is most commonly used as an anti-inflammatory agent. This study was designed to investigate the antidepressant effect of bromelain on chronic unpredictable stress (CUS)-induced depression in rat models by targeting various molecular mechanisms.

Result We studied the in silico analysis of the antidepressant potential of bromelain by docking with various proteins involved in the pathophysiology of depression. As a result of in silico studies, bromelain showed good binding energy with IL1β, 5-HT, BDNF, CREB, and TrkB. The mRNA expression of BDNF, TrkB, AKT, ERK, and IL-1β was studied by qRT-PCR. Gene expression studies showed a significant decrease in BDNF, TrkB, AKT, and ERK in chronic unpredictable stress, whereas there was a significant increase in the case of the bromelain- and fluoxetine-treated group. Since neuroinflammation is also one of the major concerns in the pathophysiology of depression, pro-inflammatory cytokines were also studied along with apoptotic markers using ELISA. ELISA results showed a significant increase in inflammatory cytokines in CUS, and it was significantly decreased in the case of the bromelain- and fluoxetine-treated group. Similarly, there was an increased concentration of pro-apoptotic protein in the CUS group, whereas it was decreased in the bromelain and fluoxetine groups.

Conclusions From the results, it is clear that bromelain exerts an antidepressive effect by preventing neuroinflammation and neurodegeneration and by enhancing neurogenesis and neuroplasticity.

Keywords Chronic unpredictable stress, BDNF, TrkB, Inflammatory markers, Apoptotic proteins, Gene expression, Molecular docking

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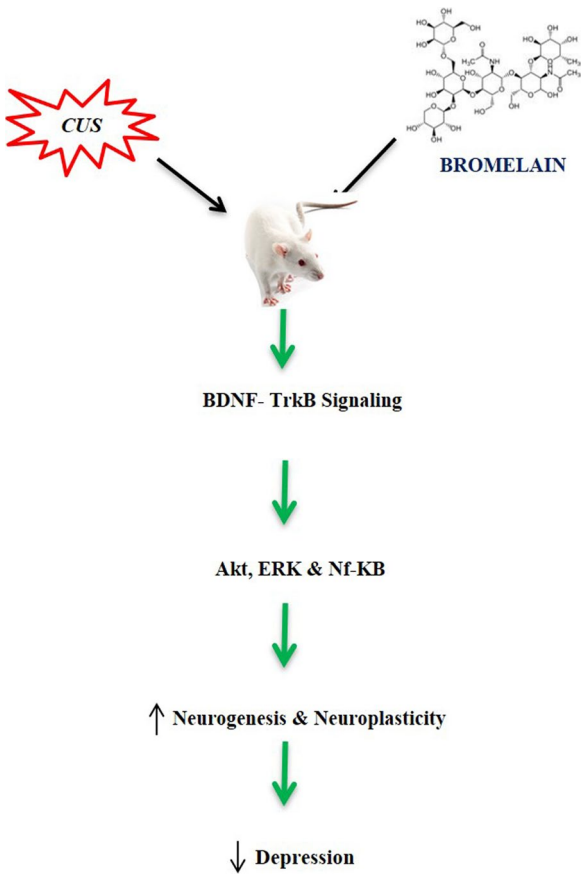
Graphical abstract



1 Background

Depression is the most common illness around the world that leads to many disabilities. The World Health Organization estimates that 350 million people worldwide suffer from depression. It is regarded as the most complex heterogeneous disorder, which is difficult to understand its pathophysiology completely; hence, it is considered as one of the biggest challenges in neuroscience. Depression significantly impairs psychosocial functioning and lowers quality of life; in worst cases, depression can also lead to suicide [1, 2]. Anxiety, anhedonia, mood swings, sleeplessness, hopelessness, and changes in appetite are some of the crippling symptoms of depression [3, 4]. Many hypotheses were put forth by researchers to study and treat depression, which include the neurotrophic hypothesis involving the reduction in neurotrophic factors, the monoamine hypothesis involving the imbalance of monoaminergic neurotransmitters and HPA axis hypothesis involving the dysfunction of the stress hormone, cortisol [5, 6]. Pathophysiology of depression involves multiple mechanisms such as oxidative stress, neuro-inflammatory cascade, decreased neurotrophic factor, aberrant cytokine production, and defective neuroplasticity [7, 8]. To study the pathophysiology of depression and to find out effective treatment strategies, studies have employed various animal models among which chronic unpredictable mild stress (CUMS) and chronic unpredictable stress (CUS) models are well established to induce depression [9, 10].

Many studies have illustrated that depression is associated with decreased neurotrophic factors, especially brain-derived neurotrophic factor (BDNF). Stress and depression reduce the expression and function of BDNF, while typical antidepressant therapy increases the peripheral BDNF expression [11, 12]. The action of BDNF is mediated through downstream activation of signal transduction by binding with its receptor tyrosine kinase B (TrkB). Overexpression of TrkB stimulates

astrocyte production, increases the number of young neurons, and also brings back basal corticosterone levels [13]. In addition, it has been reported that BDNF can increase serotonin by activating TrkB. Another study has illustrated the interconnection between serotonin, BDNF, and TrkB, and also, they can promote each other [14, 15]. Currently, selective serotonin reuptake inhibitors (SSRIs) are the most frequently prescribed antidepressants to treat depression [16]. Furthermore, 30–40% of depressive patients do not respond to the antidepressant therapy, and hence, the incidence of depression is constantly growing, and the search for safer and more effective therapies is becoming an urgent need [17]. Though fluoxetine, a selective serotonin reuptake inhibitor is an effective antidepressant, it also has many side effects including acute nausea, headaches, weight gain, and sexual dysfunction [18]. Hence, there is a need for search of a naturally occurring herbal component having antidepressant action with less or no side effects.

In the current study, we have chosen bromelain, a protease enzyme extract from the stem and core of pineapple (*Ananas comosus*), which is predominantly known for its anti-inflammatory effect. Though bromelain has an ancient origin, it has gained the attention of researchers in recent days for its vast therapeutic benefits. Traditionally, bromelain has been therapeutically used as an anti-inflammatory agent, as a modulator of tumour growth, for wound healing, and for the treatment of arthritis, episiotomy, and muscular pain [19, 20]. There are not many studies done to prove the neuroprotective effect of bromelain. This is the first study to investigate the antidepressant effects of bromelain using the gene expression analysis and enzyme-linked immunosorbent assay (ELISA) techniques. Before which this systems' bioinformatic approach was employed to uncover the atomic level interactions between bromelain and proteins involved in the molecular mechanism of pathophysiology of depression and to identify the interactions and energies of the complex. Hence, to find out the most suitable target to treat depression, molecular docking was employed.

2 Methods

2.1 Chemicals and drugs

Bromelain was procured from Sigma-Aldrich; fluoxetine was purchased from a pharmacy in Chennai, Tamil Nadu, India. ELISA kits for IL-1β (E0119Ra), NFκB (E2182Ra), BAX (E1869Ra), Bcl-2 (E1880Ra), and TNF-α (E0764Ra) were obtained from BT laboratory, Shanghai, China. For mRNA isolation, Trizol, chloroform, iso-propanol, and other chemicals were obtained from Sigma-Aldrich. Primer sequences were also bought from Sigma-Aldrich. cDNA synthesis kit was obtained from Thermo Fisher Scientific Inc. Ampliqon Red Dye Master Mix was procured from AMPLIQON, Klingenberg, Denmark. Sybr green master mix was obtained from Takara Bio Inc., Shiga, Japan.

2.2 Experimental animals

Thirty male Wistar albino rats weighing around 200–250 g were obtained from central animal house facility, Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Chennai, Tamil Nadu, India. Four animals were housed in each cage and were maintained under standard housing conditions with 12-h light/12 h dark cycle at a constant temperature of 22–24 °C with free access to food and water. The experimental protocol was approved by Institutional Animals Ethical Committee, and the experiments were carried out according to the IAEC guidelines (IAEC NO: 02/01/19).

2.3 CUS and experimental design

The animals obtained from the central animal house facility were randomly divided into five groups (n = 6/group); sample size was determined based on our previous studies. Group I: control, Group II: Bromelain (40 mg/kg bw), Group III: CUS, Group IV: CUS + Bromelain, Group V: CUS + Fluoxetine (10 mg/kg bw). The CUS procedure was conducted according to the method of Dilip et al. and Liu et al. with slight modifications, which are given in Table 1 [21, 22]. The duration of CUS was 30 days; bromelain (40 mg/kg) [23, 24] or fluoxetine (10 mg/kg) [25] was dissolved in saline and administrated orally once a day simultaneously along with CUS exposure for 30 days. The dosages of bromelain and fluoxetine were obtained from previous studies. Equal volumes of normal saline were given to rats in the control and CUS group. Gene expression analysis and cytokine estimation were performed 24 h after the last CUS exposure.

2.4 Sucrose preference test

Sucrose preference test (SPT) is the test done to measure the animal's preference for pleasure. This test was done to know whether CUS exposure has induced anxiety and depressive like behaviour in the CUS group animals. SPT was done immediately after 30 days of CUS exposure. Firstly, rats were habituated for the consumption of 1% sucrose for which the animals were presented with two bottles of sucrose solution for 48 h. Prior to the test, rats were deprived of food and water for 12 h. SPT was done by placing each rat in a separate cage, and each rat was provided with two pre-weighed bottles one containing 1% sucrose solution and the other containing drinking water. After an hour, the bottles

Table 1 The chronic unpredictable stress protocol

S. No.	Stressors	Procedure
1	Restraint stress	Restrained in Plexiglas restrainer for 2 h
2	Noise stress	Exposed to 100 dB noise for 4 h
3	Food deprivation	Deprived of food for 24 h
4	Cage rotation stress	Animal cage was placed in rotator for 1 h at 50 rpm
5	Forced swimming stress	Rats were forced to swim in a cylindrical tank which is 60cm long and 30 cm width with water to a 30 cm depth at 8 °C for 5 min
6	Sleep deprivation	Rats were placed in sleep deprivation tank for 24 h, six platforms were placed at the bottom of the tank (110×60×40 cm). The tank was filled with water at room temperature and a depth of 1.0cm below the platform surface, food and water was placed on the lid hanging over tank
7	Water deprivation	Deprived of water for 24 h

were weighed, and the amount of water or sucrose solution consumed was recorded. Percentage preference for sucrose is calculated to estimate the presence of characteristic anhedonia [26].

2.5 Sample collection

The animals were killed by decapitation, 24 h after the last CUS exposure and drug administration. The hippocampus and prefrontal cortex were dissected from the brain immediately after euthanasia, quickly frozen in dry ice and stored at – 80 °C until further analysis.

2.6 Estimation of plasma corticosterone

For estimating plasma corticosterone level, the animals were euthanized under anaesthesia with an intraperitoneal injection of 1% sodium pentobarbital (50 mg/kg) and the blood samples were collected at the jugular vein between 8 to 10 am to avoid diurnal variation. The blood samples were collected in anticoagulant-coated tubes and were centrifuged at 3000 rpm for 20 min to separate the plasma. Then, the plasma was collected and stored at − 80 °C until analysis. The plasma corticosterone levels were measured using the spectrophotometric method of Singh and Verma [27].

2.7 RNA isolation

Frozen brain tissues were homogenized in tubes with 10% Trizol. After the homogenization was complete, the tubes were stored at – 20 °C overnight. The next day the samples were thawed on ice, after thawing 1\5th of chloroform was added and centrifuged in a microfuge (10,000 rpm, 30 min) at 4 °C. After centrifugation, the aqueous phase was transferred to a fresh centrifuge tube, and then, equal volumes of isopropanol were added and stored at − 80 °C for 3 h; after 3 h, the samples were thawed on ice and centrifuged at 10,000 rpm for 10 min at 4 °C. Then, the supernatant was discarded, and the pellets were washed with ethanol and air-dried. After drying, 30 µl of DEPC water was added and short-spinned and RNA was quantified using NanoDrop 2000/2000c Spectrophotometers (Thermo Scientific). The concentration of total RNA was determined by measuring the optical density at 260 nm, and the RNA purity was assessed based on the 260 nm/280 nm ratio. The quality of the samples was determined by analysis on 1% agarose electrophoresis gel (containing Ethidium bromide) wherein Bromophenol blue was used as a staining dye to run the samples.

Table 2 Primer sequences of forward and reverse primers

Gene	Forward primer	Reverse primer	Tm (°C)	Amplicon size (bp)
GAPDH	CGGCAAGTTCAACGGCACAG	CGCCAGTAGACTCCACGACAT	60	143bp
TrkB	ACAAAGGCCTTAACAAACCT	CCACATCAAAGGCAGGAATA	60	98bp
BDNF	CCTCTGCTCTTTCTGCTGGA	GCTGTGACCCACTCGCTAAT	60	137bp
IL-1β	CCTATGTCTTGCCCGTGGAG	CACACACTAGCAGGTCGTCA	60	637bp
TNF-α	TCCCAGGTTCTCTTCAAGGGA	GGTGAGGAGCACGTAGTC GG	60	51bp
ERK	GCGTTGGTACAGAGCTCCAGAA	TGCAGCCCACAGACCAAATATC	57	289bp
AKT	TCACCTCTGAGACCGACACC	ACTGGCTGAGTAGGAGAACTGG	59	588bp

2.8 DNA oligonucleotide primers

Primers specific to rat GAPDH, BDNF, TrkB, IL-1β, TNF-α, ERK, and AKT were designed from Nucleotide BLAST(NCBI), and the melting temperature, GC content, primer dimer, and self-dimer were verified with IDT software. Primer sequences of forward and reverse primers of all the genes are given in Table 2. DNA oligonucleotide primers were custom-synthesized and obtained from Sigma-Aldrich.

2.9 First-strand cDNA synthesis and qRT-PCR

Firstly, all components of the cDNA synthesis kit were mixed and briefly centrifuged after thawing and then kept on ice. Following reagents were added into a sterile, nuclease-free tube placed on ice. 100 ng of total RNA was used as template to synthesize cDNA. RNA was first incubated with 1μL of oligo (dT), 4 μL 5X Reaction Buffer, 1 μL of RiboLock RNase Inhibitor (20 U/μL), 2 μL of 10 mM dNTP Mix, 1 μL of RevertAid RT (200 U/μL) and finally add 9 μL of nuclease-free water. Above mixture was gently mixed and spinned. After centrifugation, it was incubated for 60 min at 42 °C. The reaction was terminated by heating at 70 °C for 5 min and then briefly spinned and then proceeded with control PCR.

For control PCR amplification, synthesized cDNA was diluted in nuclease-free water. All PCR reagents were gently mixed and briefly spinned after thawing. PCR tubes were kept on ice and following reagents were added; 2 μL of diluted cDNA, 5 μL of 10X PCR buffer, 1 μL of 10 mM dNTP Mix (0.2 mM each), 3 μL of 25 mM MgCl2, 1.5 μL of forward GAPDH primer, 1.5 μL of reverse GAPDH primer, 0.5 μL of Taq DNA polymerase (5 U/μL) and 35.5 μL of nuclease-free water were added to make up a volume of 50 μL. The reaction mixture was short spin and PCR was performed in a thermal cycler for 40 cycles (94 °C for 30 s, 58 °C for 30 s,72 °C for 45 s) after an initial denaturation step at 94 °C, 3 min to activate the DNA polymerase. 5–10 μL of the PCR product was loaded on 1% agarose gel with ethidium bromide. A distinct 496 bp PCR product was visible, indicating that the synthesized cDNA has no contamination.

2.10 Real-time quantitative qRT-PCR

Amplification of reference genes was carried out using 5 μL of 1xSYBR green master mix (Takara Bio), 0.5 μL of forward and reverse primers, 100 ng of cDNA and nuclease-free water, in a total volume of 10 μL. Reactions were performed in an optical 96-well plate, using a QuantStudio™ 3 Real-Time PCR (Applied Biosystems). After an initial denaturation step at 95 °C for 10 min, amplification was performed in 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 40 s, and extension at 72 °C for 40 s. Product fluorescence was detected at the end of the elongation cycle. All melting curves exhibited a single sharp peak at the expected temperature. Ct (cycle threshold) values were calculated by the QuantStudio™ 3 data analysis software (Applied Biosystems) from fluorescence readings. The amplified products were separated by electrophoresis on 2% agarose gel and identified by ethidium bromide staining. Specificity was confirmed by the size of the amplified products with reference of 100 bp DNA ladder, and the band intensities were quantified by Gel documentation systems.

2.11 Analysis of pro-inflammatory cytokines and apoptosis-related protein

The levels of the pro-inflammatory mediators, IL-1β (E0119Ra), TNF-α (E0764Ra) and NFκB (E2182Ra) and apoptotic markers, BAX (E1869Ra), Bcl-2 (E1880Ra) in prefrontal cortex, hippocampus and amygdala were assessed using ELISA kit (BT laboratory, Shanghai, China) according to the manufacturer's instructions. The results are reported as picograms per millilitre or milligram of protein per litre.

2.12 Estimation of serotonin using spectrophotometric method

Serotonin levels were measured using spectrophotometric method of Schlumpf. For the estimation of serotonin, to 200 μL of tissue sample 250 μL of O-phthaldehyde (OPT) reagent was added. The mixture was heated at 100 °C for 10 min and then cooled to room temperature. The colour developed was read at 360–470 nm in the spectrophotometer. Tissue blank for was prepared by adding 250 μL of concentrated HCI without OPT reagent. Internal standard was prepared by taking 500 μg/mL of serotonin and making a concentration gradient of 25, 50, 75 and 100 μg. The concentration of the neurotransmitters was expressed in μg per gram of wet tissue [28, 29].

2.13 Molecular docking

To study the atomic level interactions between the ligand and proteins, BIOVIA Discovery studio simulation software was used. Minimization, C-Docker and analysis ligand pose, modules were used to study the complete interpretation of interaction and energies of the complex.

As a start, CHARMm force field applied to the ligand, bromelain (collected from pubchem) and five proteins, 5HT, BDNF, CREB, IL-1β, and TrkB in the pre-preparation of docking to calculate and correct the errors in atomic levels. In the second step, energy of molecules was minimized to near the global minima using the smart minimizer algorithm of spherical cutoff method with the

Table 3 C-Docker protocol settings for 4COO and 3COG with Simvastatin (Standard)

Protocol	Setting
Input receptor	5HT, BDNF, CREB, IL-1β, and TrkB
Input ligands	Bromelain
Top hits	10
Pose cluster radius	0.1
Random conformations	10
Dynamics steps	1000
Dynamics target temperature	1000
Include electrostatic interactions	TRUE
Orientations to refine	10
Maximum bad orientations	800
Orientation vdW energy Threshold	300

5000 maximum steps, 0.1 RMS gradient and 14.0 Å non-bonded radius. Further, active site of the protein selected based on the PDB ligand and C-Docker algorithm was used with the following condition to predict the interaction between ligand and molecule (Table 3).

2.14 Statistical analysis

Data analysis was performed using GraphPad Prism version 9.0 for Windows (GraphPad Software, La Jolla California USA). The data are presented as mean ± standard deviations, and statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. Data were considered statistically significant when $p < 0.05$. Confidence interval (CI) 95% was calculated according to the designed model.

3 Results

The animals were tested for the presence of CUS-induced depression by evaluating sucrose preference test and corticosterone levels. Animals of all the groups were stable in the course of treatment. No animals died during the model establishment or intervention period. Fluoxetine- and bromelain-treated groups showed positive results compared with the CUS group, which indicated the improvement of depression-like behaviour of rats after both treatments ($p < 0.05$).

3.1 Sucrose preference test

Sucrose preference test is done to confirm the presence of anhedonia, which is a crucial characteristic of depression. In the sucrose preference test, the percentage of sucrose consumed by the chronic unpredictable stress group was significantly decreased than that of the other groups with a P value ≤ 0.0001, suggesting the existence of characteristic anhedonia, which is the marker for depression. When compared to the chronic unpredictable stress group, there was a significant rise in the sucrose preference percentage in the group treated with bromelain and fluoxetine (known antidepressants) with a P value ≤ 0.0001 and with a F value of F (4, 15) = 444.0.

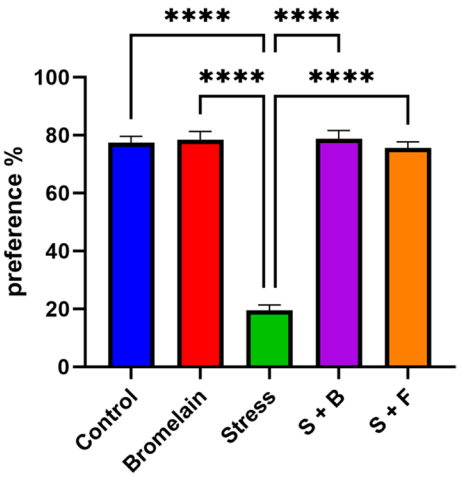


Fig. 1 Percentage preference for sucrose in control, Bromelain, stress, stress + Bromelain, stress + fluoxetine group. All data are presented as the mean ± SD. $n = 6$ per group. P Value—" > 0.05" (NS), " ≤ 0.05" (*), " ≤ 0.01" (**), ≤"0.001" (***), " ≤ 0.0001" (****)

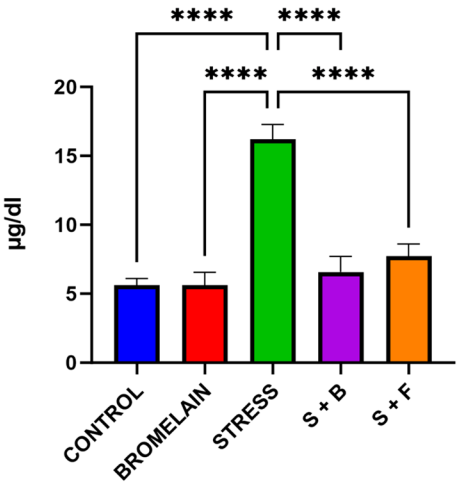


Fig. 2 Plasma corticosterone level in control, Bromelain, stress, stress + Bromelain, stress + fluoxetine were analysed by spectrophotometry. All data are presented as the mean ± SD. $n = 6$ per group. P Value—" > 0.05" (NS), " ≤ 0.05" (*), " ≤ 0.01" (**), ≤"0.001" (***), " ≤ 0.0001" (****)

This indicates that the administration of bromelain has successfully reinstated the inclination towards sucrose (Fig. 1).

3.2 Corticosterone estimation

A significant increase in plasma corticosterone levels has been observed in the chronic unpredictable stress group ($P \leq 0.0001$, F (4, 25) = 140.6) as a coping mechanism for the exposure to stressors. This is yet another indicator of depression and stress exposure. Treatment with bromelain and fluoxetine has effectively restored the negative feedback mechanism of the HPA axis as well as the elevated corticosterone levels in response to chronic unpredictable stress (Fig. 2).

3.3 The mRNA expression of BDNF-TrkB signalling pathway associated genes and the genes of pro-inflammatory cytokine

The RT-PCR displayed mRNA expression of BDNF (Fig. 4c and d), TrkB (Fig. 4a and b), IL-1β (4.e and 4.f), TNF-α (4g and 4h), ERK (4i and 4j), and AKT (4k and 4l) in the prefrontal cortex and hippocampus of all groups. Figure 3 shows the relative fold change of TrkB, BDNF, IL-1β, TNF-α, ERK, and AKT expression in prefrontal cortex and hippocampus of control, Bromelain, stress, stress + Bromelain, stress + fluoxetine group. The relative fold change of TrkB (prefrontal cortex) depict that F (4, 10) = 15.19 and $P \leq 0.01$ for control, stress + bromelain and stress + fluoxetine groups in comparison

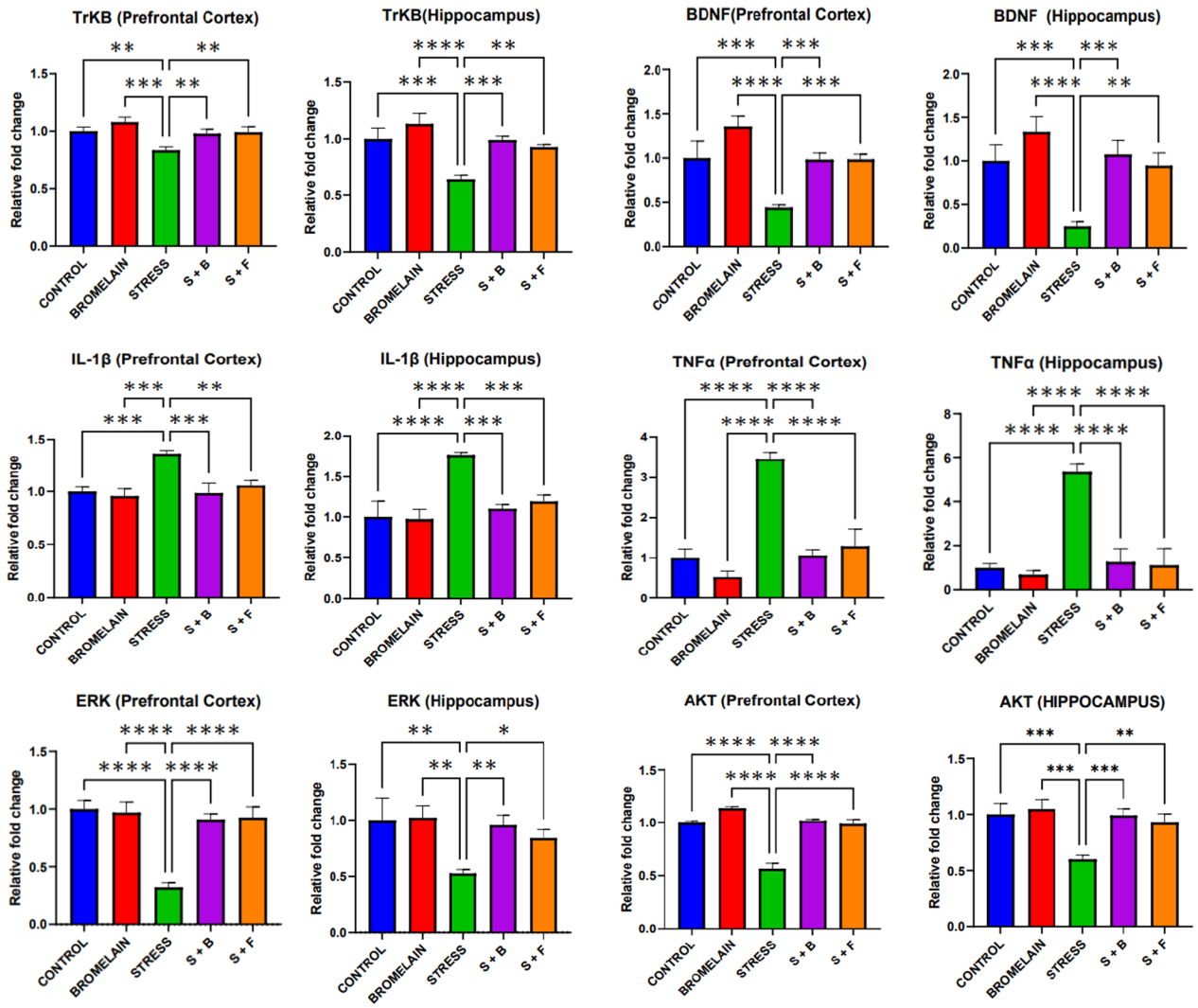


Fig. 3 The relative fold change of TrkB, BDNF, IL-1β, TNF-α, ERK and AKT expression in Prefrontal cortex and hippocampus of control, Bromelain, stress, stress + Bromelain, stress + fluoxetine group. All data are presented as the mean ± SD. $n = 6$ per group. P Value—"> 0.05" (NS), "≤ 0.05" (*), "≤ 0.01" (**), ≤"0.001" (***), "≤ 0.0001" (****)

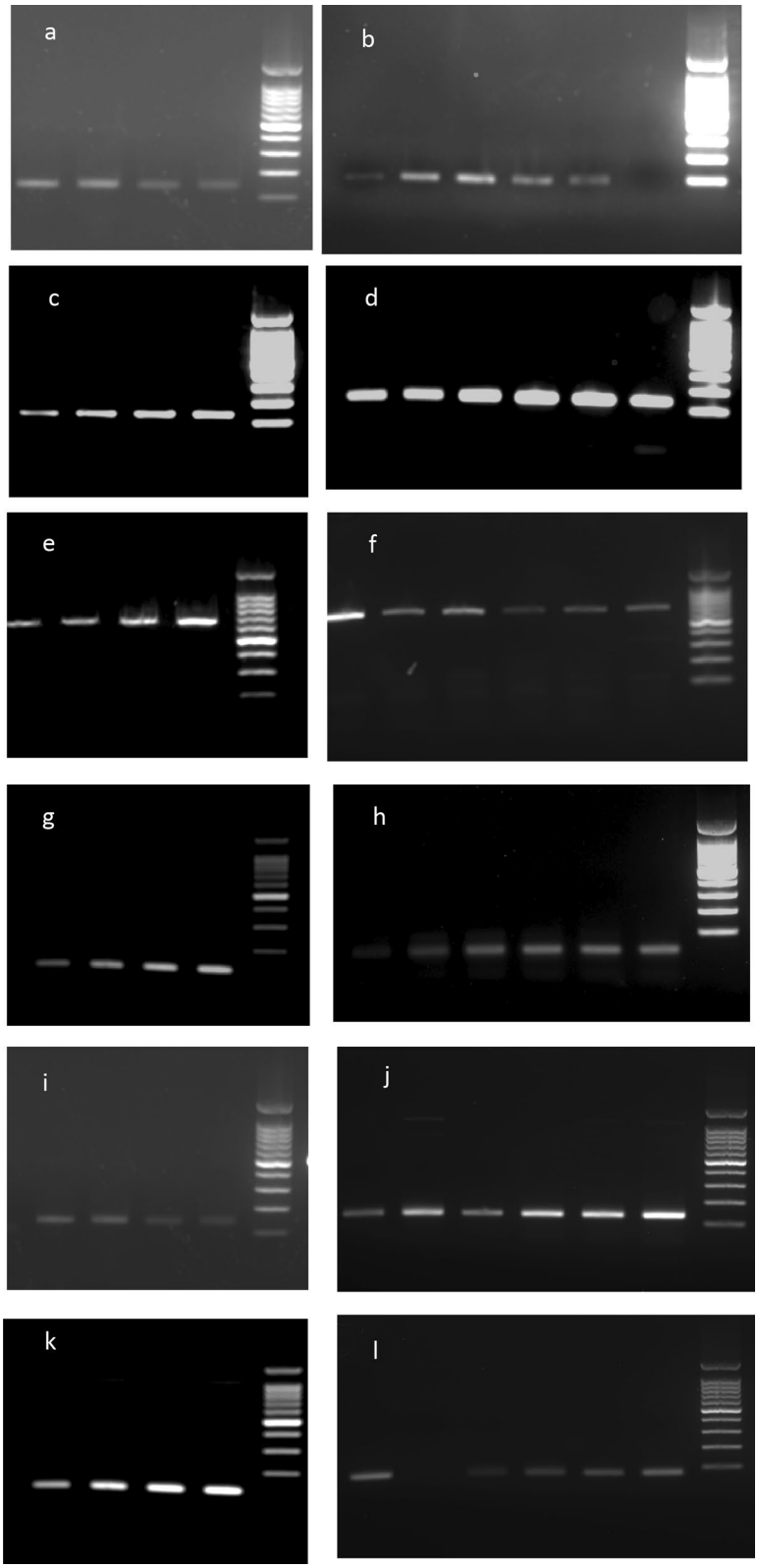


Fig. 4 PCR amplification of TrkB, BDNF, IL-1β, TNF-α, ERK and AKT gene fragment from rat Prefrontal cortex and hippocampus. Wherein image 2a and 2b indicating TrkB amplification, image 2c and 2d indicating BDNF amplification, image 2e and 2f indicating IL-1β amplification, image 2g and 2h indicating TNF-α amplification, image 2i and 2j indicating ERK amplification, 2k and 2l indicating AKT amplification

with stress group, $P \leq 0.001$ for bromelain group in comparison with stress group. The relative fold change of TrkB (hippocampus) depicts that F (4, 10) = 25.11 and $P \leq 0.01$ for stress + fluoxetine group in comparison with stress group, $P \leq 0.001$ for control and stress + bromelain groups in comparison with stress group, $P \leq 0.0001$ for bromelain group in comparison with stress group. The relative fold change of BDNF(prefrontal cortex) depicts that F (4, 10) = 26.67 and $P \leq 0.001$ for control, stress + bromelain and stress + fluoxetine groups in comparison with stress group, $P \leq 0.0001$ for bromelain group in comparison with stress group. The relative fold change of BDNF(hippocampus) depicts that F (4, 10) = 21.44 and $P \leq 0.01$ for stress + fluoxetine group in comparison with stress group, $P \leq 0.001$ for control and stress + bromelain groups in comparison with stress group, $P \leq 0.0001$ for bromelain group in comparison with stress group. IL-1β (prefrontal cortex) depicts that F (4, 10) = 20.92 and $P \leq 0.001$ for control, bromelain and stress + bromelain groups in comparison with stress group, $P \leq 0.01$ for stress + fluoxetine group in comparison with stress group. The relative fold change of IL-1β(hippocampus) depicts that F (4, 10) = 25.15 and $P \leq 0.0001$ for control and bromelain groups in comparison with stress group, $P \leq 0.001$ for stress + bromelain and stress + fluoxetine group in comparison with stress group. The relative fold change of TNF-α (prefrontal cortex) depicts that F (4, 10) = 65.53 and $P \leq 0.0001$ for control, bromelain, stress + bromelain and stress + fluoxetine groups in comparison with stress group. The relative fold change of TNF-α (hippocampus) depicts that F (4, 10) = 51.87and $P \leq 0.0001$ for control, bromelain, stress + bromelain and stress + fluoxetine groups in comparison with stress group. The relative fold change of ERK (prefrontal cortex) depicts that F (4, 10) = 44.68 and $P \leq 0.0001$ for control, bromelain, stress + bromelain and stress + fluoxetine groups in comparison with stress group. The relative fold change of ERK (hippocampus) depicts that F (4, 10) = 9.528 and $P \leq 0.01$ for control, bromelain and stress + bromelain groups in comparison with stress group, $P \leq 0.05$ for stress + fluoxetine group in comparison with stress group. The relative fold change of AKT (prefrontal cortex) depicts that F (4, 10) = 17.94 and $P \leq 0.0001$ for control, bromelain, stress + bromelain and stress + fluoxetine groups in comparison with stress group. AKT (hippocampus) depicts that F (4, 10) = 124 and $P \leq 0.001$ for control, bromelain and stress + bromelain groups in comparison with stress group, $P \leq 0.01$ for stress + fluoxetine group in comparison with stress group. Overall, the above P values indicate that the gene expression of BDNF, TrkB, ERK and AKT in the prefrontal cortex and hippocampus of CUS group decreased significantly compared to the control group, whereas expression of BDNF, TrkB, ERK, and AKT was significantly increased in CUS + bromelain-treated group compared with CUS group, but there were no significant changes when compared to other groups (Control, Bromelain and CUS + fluoxetine group). These results are indicating the efficacy of bromelain in promoting neurogenesis. The gene expression of IL-1β and TNF-α in the prefrontal cortex and hippocampus of CUS group was significantly increased compared to the control group, which indicates the characteristic neuroinflammation in depression. There is a significant decrease in IL-1β and TNF-α expression in CUS + bromelain-treated group compared with CUS group which indicates anti-inflammatory effect of bromelain; hence, bromelain could efficiently prevent neuroinflammation (Fig. 4).

3.4 Effect of bromelain on pro-inflammatory cytokine and apoptosis mediators using ELISA kit

Estimation of IL-1β, TNF-α and NFκB by ELISA revealed that CUS has significantly increased the concentration of pro-inflammatory cytokine in the prefrontal cortex, hippocampus, and amygdala (Fig. 5). NFκB levels in prefrontal cortex of CUS group are significantly increased with a F value of F (4, 10) = 26.83 and P value of $P \leq 0.0001$ in comparison with control, bromelain and stress + bromelain groups and with a P value of $P \leq 0.001$ in comparison with stress + fluoxetine group. NFκB level in hippocampus of CUS group is significantly increased with a F value of F (4, 10) = 12.50 and P value of $P \leq 0.01$ in comparison with control, bromelain stress + bromelain and stress + fluoxetine groups. NFκB level in amygdala of CUS group is significantly increased with a F value of F (4, 10) = 16.96 and P value of $P \leq 0.001$ in comparison with control, bromelain and stress + bromelain groups and with a P value of $P \leq 0.01$ in comparison with stress + fluoxetine group. Similarly IL-1β level in prefrontal cortex of CUS group is significantly increased with a F value of F (4, 10) = 38.73 and P value of ≤ 0.0001 in comparison with control, bromelain stress + bromelain and stress + fluoxetine groups. IL-1β level in hippocampus of CUS group is significantly increased with a F value of F (4, 10) = 26.47 and P value of ≤ 0.0001 in comparison with control, bromelain and stress + bromelain groups and with a P value of ≤ 0.001 in comparison with stress + fluoxetine group. IL-1β level in amygdala of CUS group is significantly increased with a F value of F (4, 10) = 13.75 and P value of ≤ 0.001 in comparison with bromelain group and with a P value of ≤ 0.01 in comparison with control, stress + bromelain, stress + fluoxetine group. TNF-α level in prefrontal cortex of CUS group is significantly increased with a F value of F (4, 10) = 15.05 and P value of ≤ 0.001 in comparison with control, bromelain and stress + bromelain groups and with a P value of ≤ 0.01 in comparison with stress + fluoxetine group.

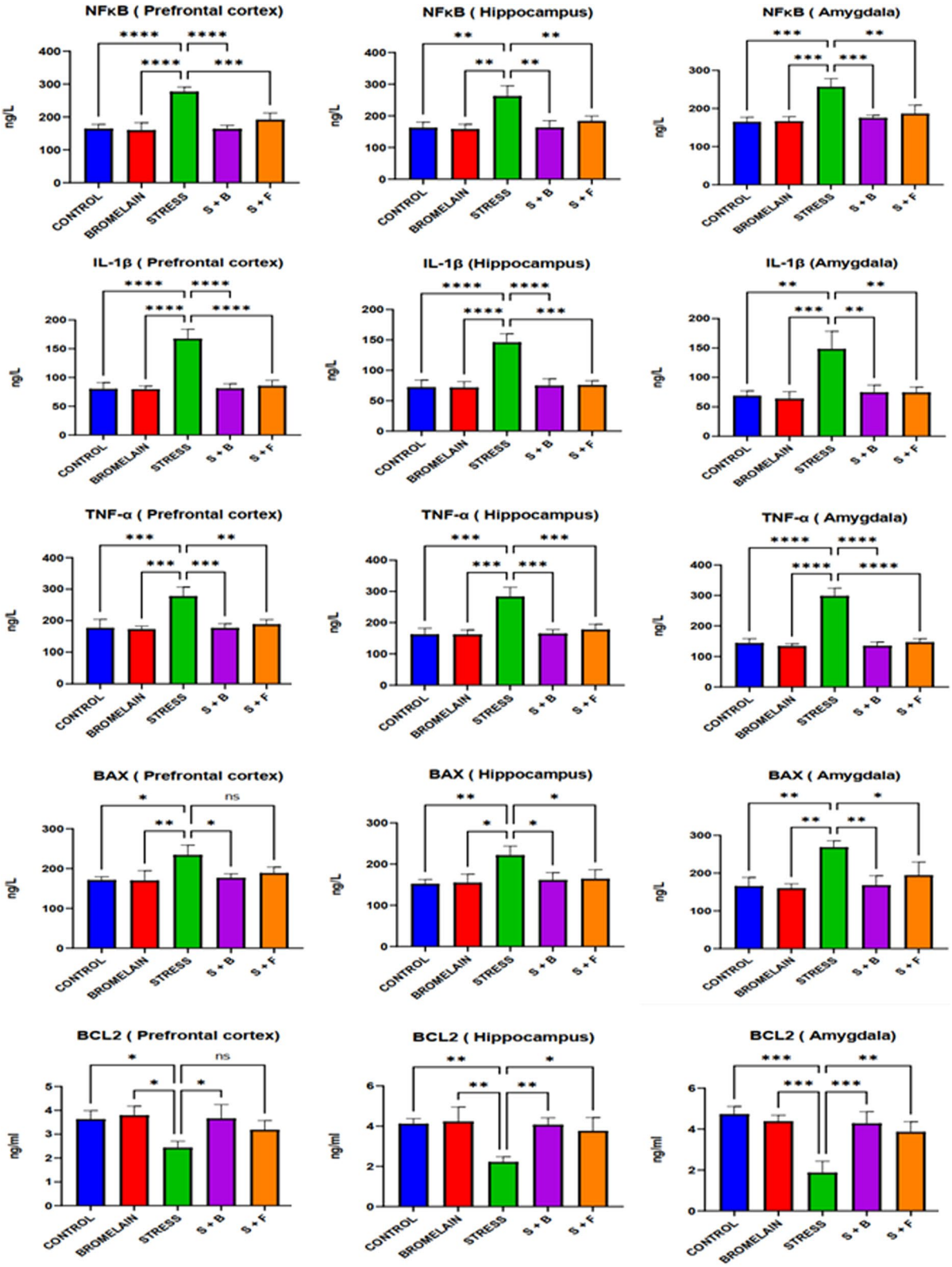


Fig. 5 NFκB, IL-1β, TNF-α, BAX and Bcl-2 protein levels in Prefrontal cortex, hippocampus and amygdale of control, Bromelain, stress, stress + Bromelain, stress + fluoxetine were analysed by ELISA. All data are presented as the mean ± SD. *n* = 6 per group. *P* Value—" > 0.05" (NS), " ≤ 0.05" (*), " ≤ 0.01" (**), ≤ "0.001" (***), " ≤ 0.0001" (****)

TNF-α level in hippocampus of CUS group is significantly increased with a *F* value of *F* (4, 10) = 21.84 and *P* value of ≤ 0.001 in comparison with control, bromelain stress + bromelain and stress + fluoxetine groups. TNF-α level in amygdala of CUS group is significantly increased with a *F* value of *F* (4, 10) = 64.72 and *P* value of ≤ 0.0001 in comparison with control, bromelain stress + bromelain and stress + fluoxetine groups. These increases were attenuated by treatment with bromelain, which was similar to that of fluoxetine treatment. Analysis of apoptotic markers, BAX and Bcl-2 has revealed that there was significant increase in the concentration of pro-apoptotic protein, BAX in all the 3 regions of CUS group compared to control with a *P* value of ≤ 0.01 in prefrontal cortex and amygdala and with a *P* value of ≤ 0.05 in hippocampus, indicating the characteristic neurodegeneration in depression, whereas in CUS + Bromelain and CUS + Fluoxetine-treated group there was significant decrease in BAX concentration and almost reached the concentration levels in control and Bromelain alone group with a *P* value of ≤ 0.05 except for prefrontal cortex of CUS + Fluoxetine-treated group which showed no significant change in BAX level when compared to CUS group. The corresponding *F* values of BAX are *F* (4, 10) = 6.767 in prefrontal cortex, *F* (4, 10) = 6.879 in hippocampus and *F* (4, 10) = 10.98 in amygdala. Similarly, concentration of anti-apoptotic protein, Bcl-2 in prefrontal cortex was significantly decreased in CUS group compared to control, bromelain, CUS + bromelain with a *F* value of *F* (4, 10) = 5.616 and *P* value of ≤ 0.05 except for CUS + Fluoxetine group which showed no significant changes. Bcl-2 level in hippocampus was significantly decreased in CUS group compared to control, bromelain, CUS + bromelain with a *F* value of *F* (4, 10) = 8.723 and *P* value of *P* ≤ 0.01 and with a *P* value of *P* ≤ 0.05 in comparison with CUS + Fluoxetine group. Similarly Bcl-2 level in amygdala was significantly decreased in CUS group compared to control, bromelain, CUS + bromelain with a F value of F (4, 10) = 17.48 and *P* value of ≤ 0.001 and with a *P* value of ≤ 0.01 in comparison with CUS + Fluoxetine group. Hence , from the above findings it is clear that bromelain has effectively attenuated the neuro-inflammatory and neurodegenerative changes brought about by chronic unpredictable stress exposure.

3.5 Estimation of serotonin

One of the major contributors in the pathophysiology of depression is serotonin. Serotonin levels were estimated in prefrontal cortex, hippocampus, and amygdala because previous reports are indicating higher alterations in these regions in depression. Comparing the CUS group to the other groups, there was a significant drop in serotonin levels (Fig. 6), which is an indication of anxiety and depression. This alteration in CUS group is restored by bromelain treatment as that of fluoxetine with a *F* value of *F* (4, 25) = 15.46 and *P* value of ≤ 0.0001 in prefrontal cortex, with a *F* value of *F* (4, 25) = 14.53 and *P* value of ≤ 0.0001 in hippocampus, with a *F* value of *F* (4, 25) = 14.43 and *P* value of ≤ 0.0001 in amygdala.

3.6 Molecular docking of bromelain on the active site of serotonin

The force field and minimization algorithm run on the protein 5HT (Serotonin) resulted the stable energy level of − 69,678.6 kcal/mol. The define binding pocket algorithm developed the binding pocket at the coordinates

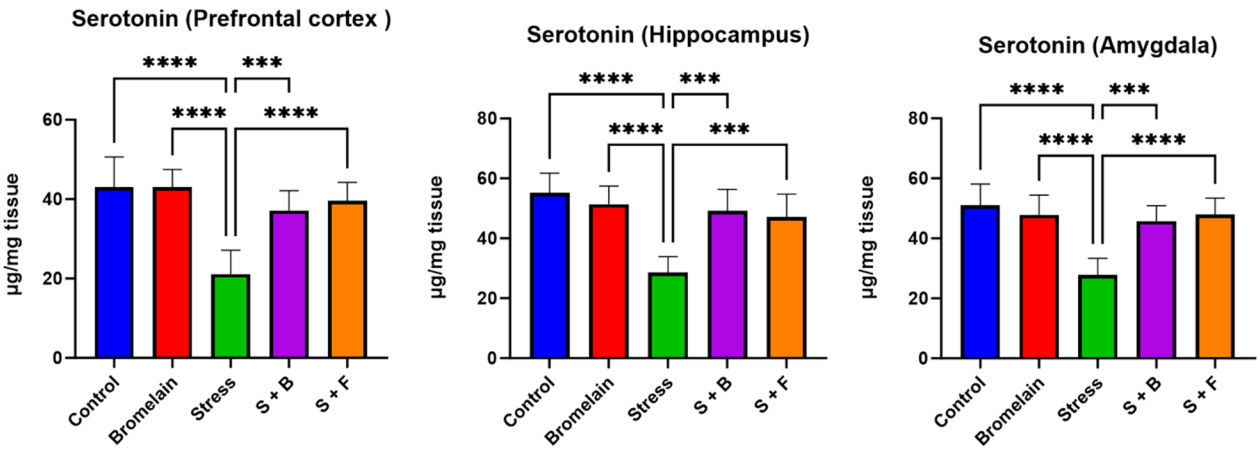


Fig. 6 Serotonin(5HT) levels in Prefrontal cortex, hippocampus and amygdale of control, Bromelain, stress, stress + Bromelain, stress + fluoxetine were analysed by spectrophotometry. All data are presented as the mean ± SD. *n* = 6 per group. *P* Value—"> 0.05" (NS), "≤ 0.05" (*), "≤ 0.01" (**), ≤ "0.001" (***), "≤ 0.0001" (****)

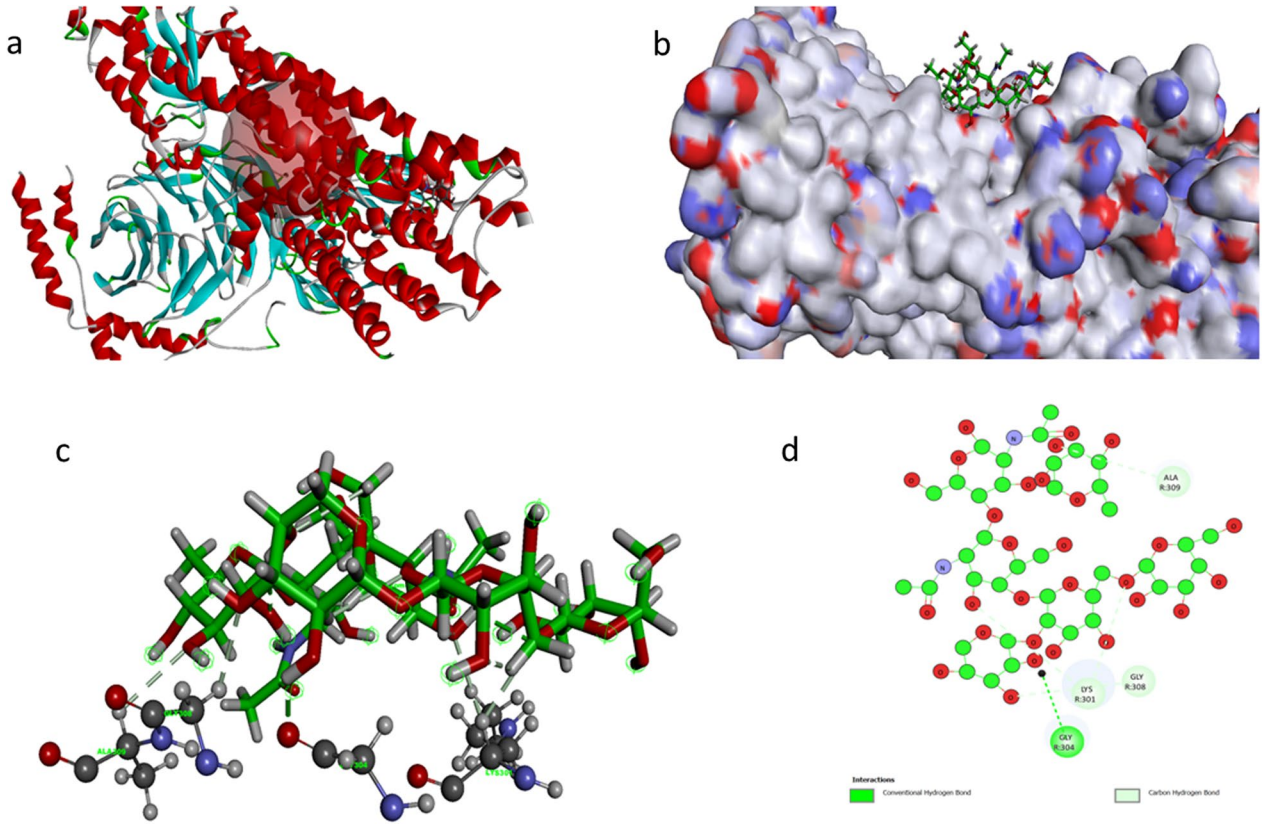


Fig. 7 **a** Secondary structure of 5HT-Receptor with the binding pocket. **b** Surface binding of bromelain molecule on the protein 5HT-Receptor. **c** Interaction of active site amino acids of the protein 5HT with bromelain molecule (3D view). **d** 2D view of the molecule

Table 4 Various energies and docking energy of the bromelain molecule with 5 proteins

Name	Initial Potential Energy	Initial RMS gradient	Electrostatic Energy	Final Potential Energy	vdW energy	Final RMS gradient	Dock energy
5 HT	− 26,068.7	117.015	− 71,416.8	− 69,678.6	− 7,594.3	1.19648	
Bromelain	567.956	35.6451	− 173.058	9.75461	− 15.5311	0.94098	− 37.3561
BDNF	− 6,083.12	32.0812	− 13,700.8	− 13,447.2	− 1,391.46	1.07727	
Bromelain	567.956	35.6451	− 173.058	9.75461	− 15.5311	0.94098	− 33.6162
CREB	2.4846	8.6024	− 16,935.4	− 14,864.7	− 973.467	1.65673	
Bromelain	567.956	35.6451	− 173.058	9.75461	− 15.5311	0.94098	− 23.265
1L-1β	35,589.6	5,059.84	− 53,605.8	− 52,401.5	− 5,344.93	1.06102	
Bromelain	567.956	35.6451	− 173.058	9.75461	− 15.5311	0.94098	− 36.9467
TrkB	− 6,958.09	294.389	− 18,159.6	− 18,047	− 2,075.86	1.05823	
Bromelain	567.956	35.6451	− 173.058	9.75461	− 15.5311	0.94098	− 64.5259

of X-81.593, y-119.033 and z-96.2501 with the radius of r-10.9259 A (Fig. 7a). The CHARMm-based docking protocol run resulted in the best binding possess of the bromelain molecule on the surface of serotonin (Fig. 7b). The interaction pattern analysis of bromelain molecule with 5HT protein developed two kinds of interactions such as conventional and carbon hydrogen bonds. The Ala 309, Lys 301 and Gly 308 formed the network with the Oxygen atom of the bromelain molecule. Further the Gly303 formed one conventional Hydrogen bond with the Hydroxyl group hydrogen of bromelain (Fig. 7c, d). This interaction and other atomic bond energies of active site binding of molecule produce a total energy of − 37.3561 kcal/mol (Table 4).

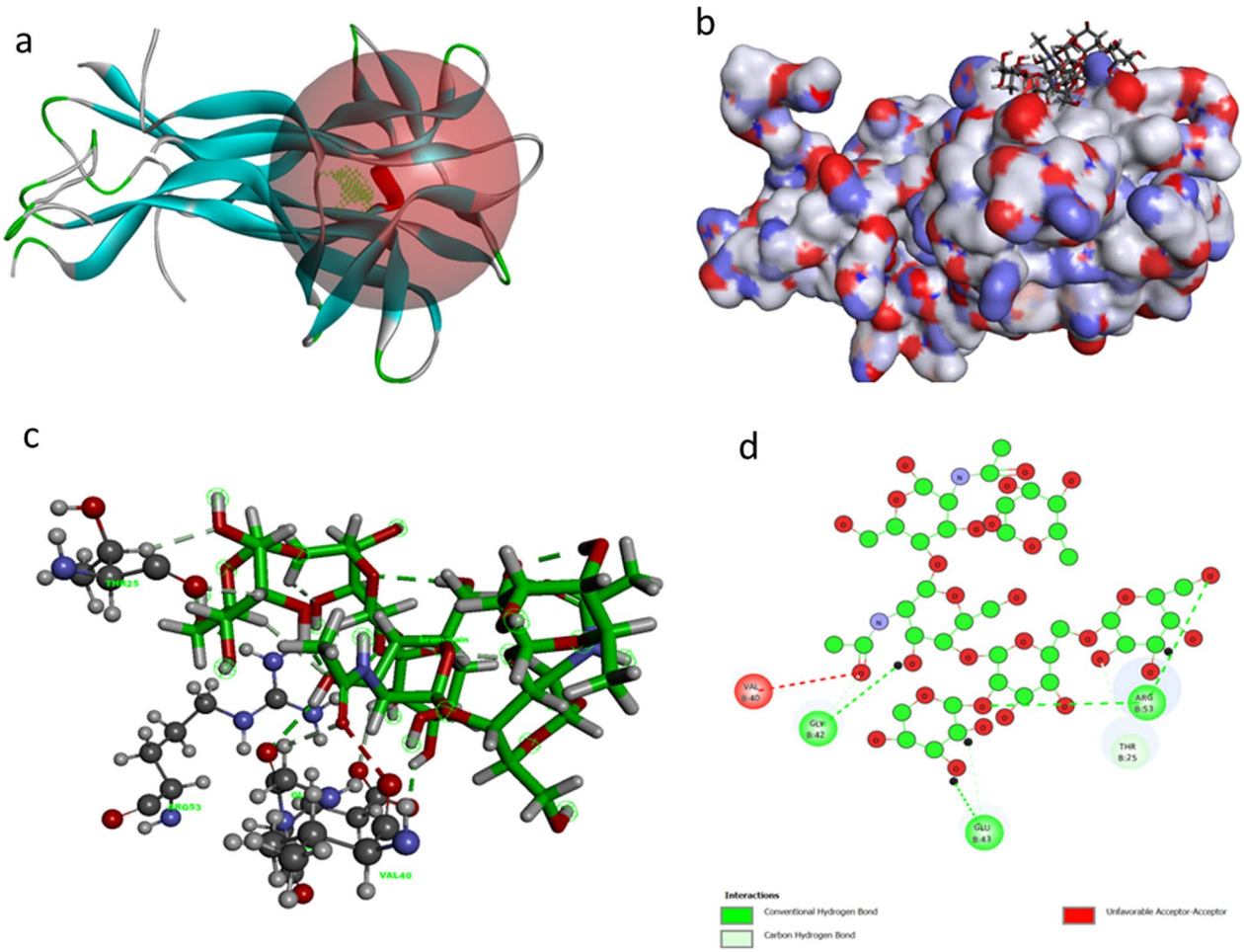


Fig. 8 **a** Secondary structure of BDNF with the binding pocket. **b** Surface binding of bromelain molecule on the protein BDNF. **c** Interaction of active site amino acids of the protein BDNF with bromelain molecule (3D view). **d** 2D view of the molecule

3.7 Molecular docking of bromelain on the active site of BDNF

The protein BDNF was minimized to the energy of − 13,447.2 kcal/mol to generate a stable protein for the docking process, and the binding pocket was defined in the coordinates of x-(− 15.3191), y-34.581, z-9.05769 and radius of 12 A (Fig. 8a). Energy minimized protein was docked with bromelain by the CDOCKER algorithm, which generated the best configured pose of the bromelain on the surface of BDNF. Bromelain showed good binding on the hydrophobic region of BDNF (Fig. 8b). Bromelain binds to BDNF with the energy of -33.6162 kcal/mol; this energy was developed by four hydrogen bonds with Gly42, Glu 43 and Arg 53. Further, Thr25 formed carbon hydrogen bond and Val40 produced unfavourable acceptor—acceptor repulsion with the ketonic (−C=O) group of the molecule not making suitable for inhibition of this protein (Fig. 8c, d).

3.8 Molecular docking of bromelain on the active site of CREB

In the first, energy of CREB was minimized and the stable protein potential energy found to be-14,864.7 kcal/mol. The ligand binding site for the protein was found in the coordinates of x,y,z (73.0347, 34.4396, 53.2316) with the radius of 12 A. Bromelain molecule was docked using the CHARMm algorithm, and the result showed that the molecule binds well between the space of protein and the DNA interacting position (Fig. 9a, b). The dock energy of the molecule was found to be − 23.265 kcal/mol, generated due to the conventional hydrogen bond, carbon hydrogen bond and π-Alkyl bond. The conventional hydrogen networks Lys 285, Arg286, Arg 289 and 2 nucleosides (DA4, DA5), specifically the Arg 289, played a major role in the formation of more binding complex with 5 conventional hydrogen bonds with the various oxygen atoms of the bromelain. Also, Arg 289 created the carbonic hydrogen bonds and the $-CH_3$ present in the

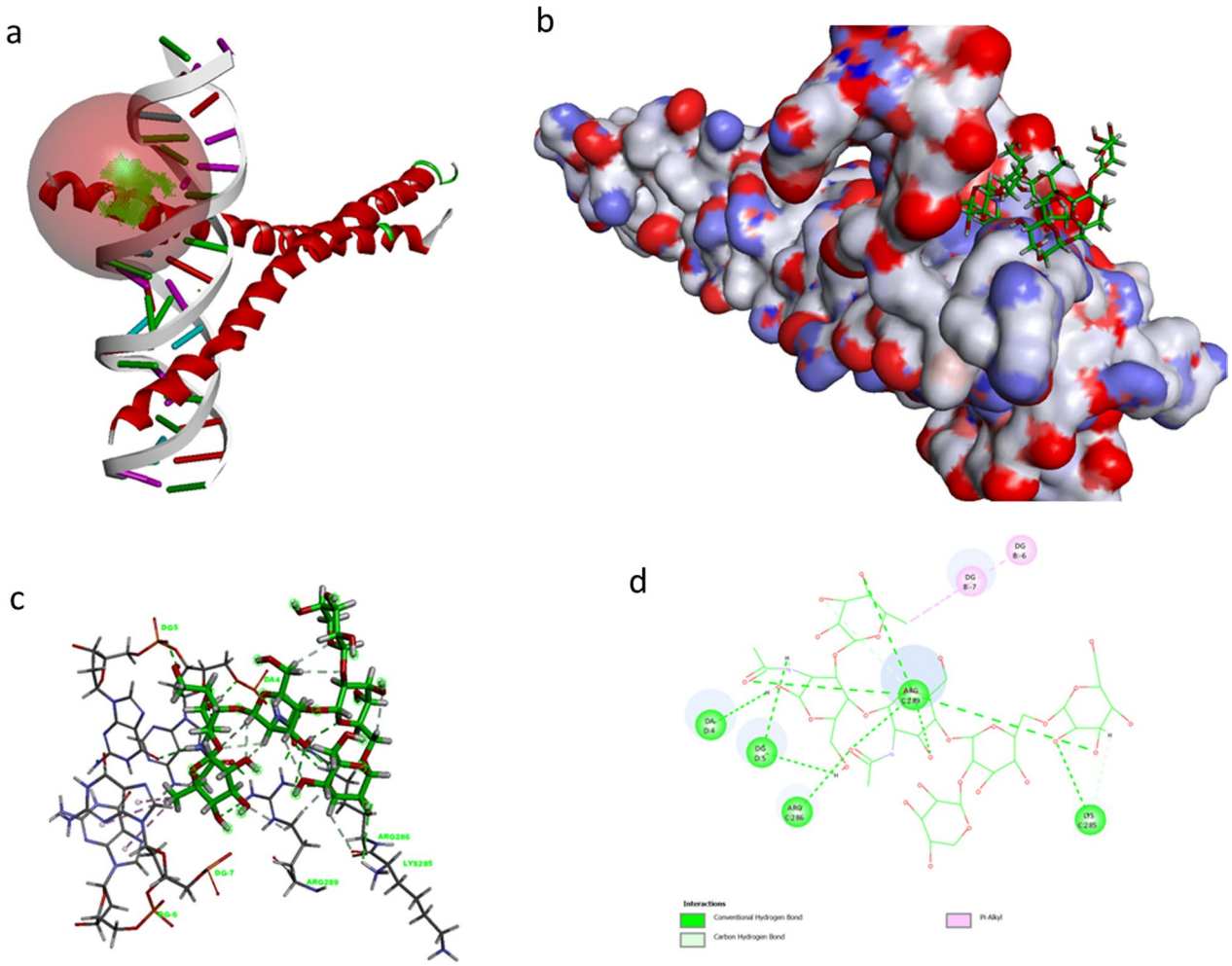


Fig. 9 **a** Secondary structure of protein CREB with the binding pocket, **b** Surface binding of bromelain molecule on the protein CREB, **c** Interaction of active site amino acids of the protein CREB with bromelain molecule (3D view), **d** 2D view of the molecule

bromelain molecule formed two π-Alkyl interaction with the DNA nucleotides of DG6, 7 (Fig. 9c, d). These overall interactions made the molecule for better interaction.

3.9 Molecular docking of bromelain on the active site of IL-1β

The energy of IL-1β was minimized to − 52,401.5 kcal/mol for the docking process by smart minimizer algorithm. The binding pocket was defined by the PDB binding site information on coordinates of *x*, *y*, *z* (− 2.75622, 36.0782, 5.22528) with the radius of 12A (Fig. 10a). The CDOCKER algorithm docked the bromelain molecule on the surface of the molecule with the energy of − 36.9467 kcal/mol and most of the hydrophilic fragment of the molecule (Fig. 10b). Bromelain formed 6 conventional hydrogen bonds with the amino acids Gln 3, Thr6, Glu7, Ser26, Thr41, and Asp83 of the active site of IL-1β. Lys102 carbon formed hydrogen bond with hydroxyl oxygen of the oxane ring of the molecule. Similarly, Thr41 also formed conventional hydrogen bond with the hydroxyl group of tallied oxane ring of the bromelain (Fig. 10c, d). These interactions completely configured the bromelain molecules inside binding pocket of IL-1β leading to the complete denaturation and inhibition.

3.10 Molecular docking of bromelain on the active site ofTrkB

Energy of TrkB protein was minimized to-18047kcl/mol, and the binding pocked was found at the coordinates of x-57.3486, y (− 0.747099), z-23.3297 and radius of 12 A (Fig. 11a, b). Bromelain molecule was binding with the energy of − 64.5259 kcal/mol on the surface of the TrkB. Further the interaction analysis of the protein showed that the molecules formed only two interactions, such as one is conventional hydrogen bond with the amino acid Tyr771. Secondly, the amino acid Gln 789 networked

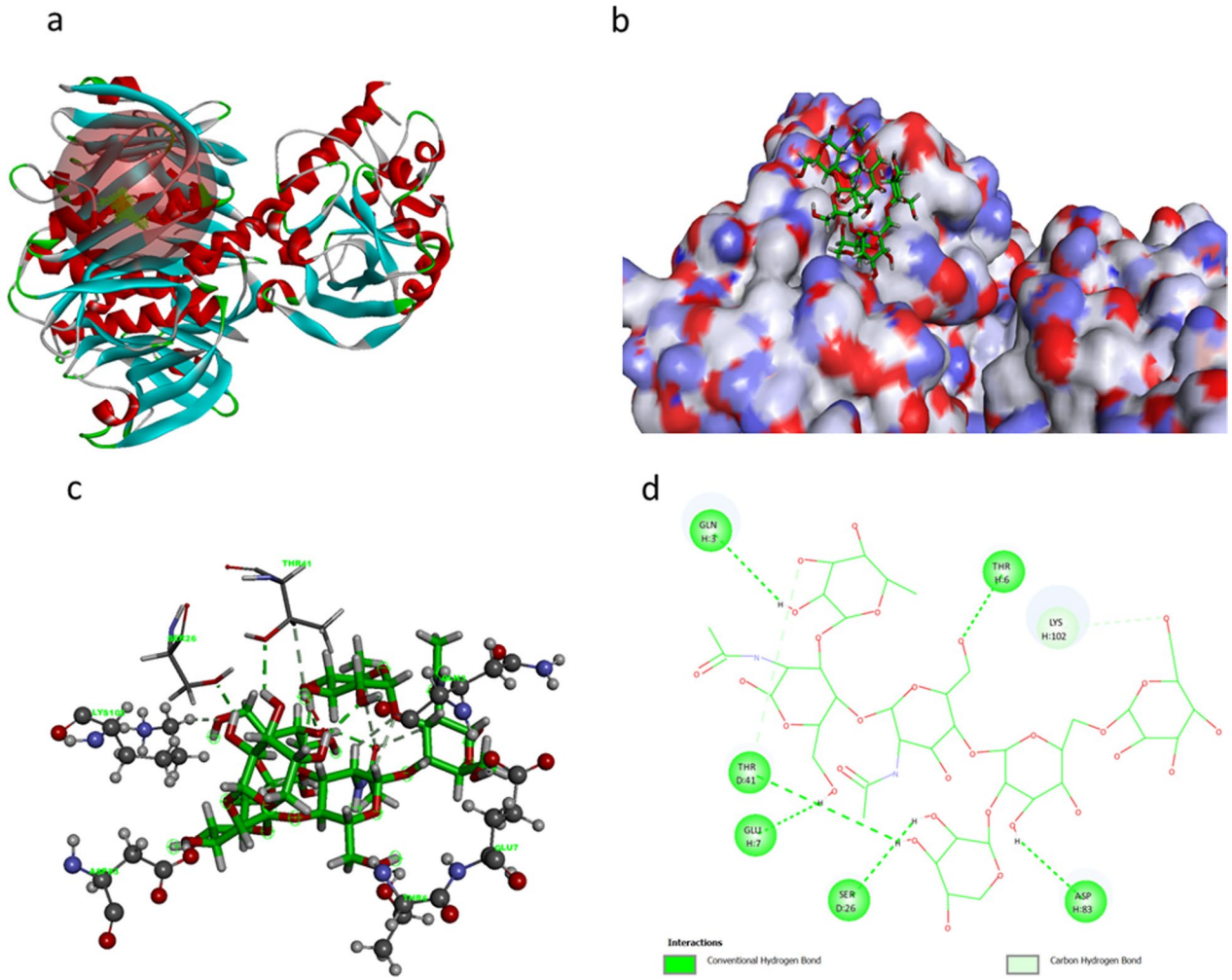


Fig. 10 **a** Secondary structure of protein IL-1β with the binding pocket, **b** Surface binding of bromelain molecule on the protein IL-1β, **c** Interaction of active site amino acids of the proteinIL-1β with bromelain molecule (3D view), **d** 2D view of the molecule

with the bromelain molecule hydrogen of aliphatic hydroxyl region (Fig. 11c, d). Less interaction made this molecule less effective against the TrkB protein.

4 Discussion

Role of the current study was to investigate the antidepressant action of bromelain in Wistar albino rats exposed to CUS by targeting the underlying molecular mechanisms. The CUS model is the modified form of CUMS, one of the most reliable models used to screen antidepressants in which animals were subjected to different stressors on a daily basis [30, 31]. Sucrose preference test and estimation of corticosterone are crucial for assessing the induction of depression and to test whether the drug exhibits antidepressant activity. Our study has demonstrated that bromelain has significantly alleviated depression-like behaviour in rats subjected to CUS. This is well proved by the mitigating effect of bromelain treatment on CUS-induced anhedonia (reduced sucrose preference). Also bromelain treatment has effectively reduced the corticosterone level, which was increased on CUS exposure. Additionally, bromelain treatment has also effectively counteracted CUS-induced neuro-inflammatory and neurodegenerative changes.

Our previous study has showed that CUS-induced oxidative changes and monoaminergic changes were attenuated by bromelain treatment. This is the first study to demonstrate the antidepressant effect of bromelain on CUS-induced depression by targeting various mechanisms. An effective drug designing involves the finding of an appropriate molecule, which is complementary to the biological receptor. And also it should bind and interact with the target protein to bring about the pharmacological actions. Hence, with the help of modern method of drug designing, molecular docking analysis of bromelain with various proteins was done with the

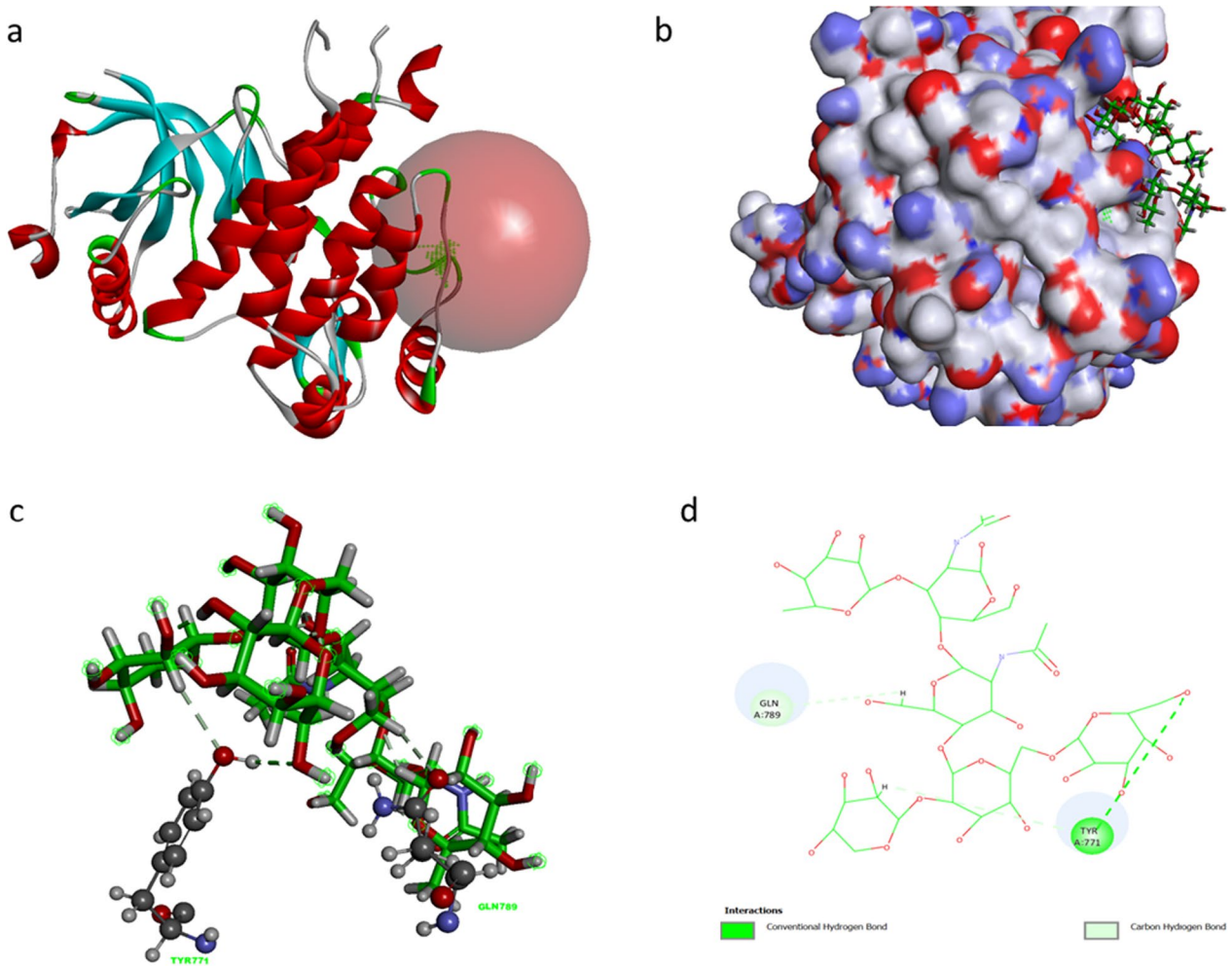


Fig. 11 **a** Secondary structure of protein TrkBwith the binding pocket, **b** Surface binding of bromelain molecule on the protein TrkB, **c** Interaction of active site amino acids of the protein TrkB with bromelain molecule (3D view), **d** 2D view of the molecule

aid of computers to know the binding abilities and the action of bromelain on different proteins before moving on to the animal experiment. The results of atomic level interactions revealed that TrkB has the highest binding energy, but since there are only two interactions between bromelain and TrkB, bromelain is less effective than TrkB. The bromelain molecule showed good binding on the hydrophobic region of BDNF. The BDNF produced an unfavourable acceptor—acceptor repulsion with the ketonic (–C=O) group of the molecule not making it suitable for inhibition of this protein, which proves that bromelain activates BDNF. In the case of 1L-1β, all the interactions have completely configured the bromelain molecules inside the binding pocket of 1L-1β leading to complete denaturation and inhibition of the protein. Finally, docking studies have revealed that bromelain binds to the active site of 5-HT receptor and activates it, which in turn can activate BDNF and TrkB. Hence, computationally bromelain is proven to be a potential drug candidate having a good binding affinity with BDNF, 1L-1β, 5HT-receptor and CREB.

Recent studies have concentrated more on the role of inflammation in the aetiology of neurodegeneration and depression [32]. It has been reported that there is increased secretion of pro-inflammatory cytokines such as IL-1β and TNF-α in the brain of patients with major depressive disorder [33]. In correlation to this, in our current study rats subjected to CUS showed elevated levels of these cytokines; however, the levels in the bromelain-treated group were significantly decreased than those in the CUS group and were similar to the levels in rats treated with the standard antidepressant fluoxetine. We further studied whether clinically effective antidepressants would influence the expression of the apoptotic genes. We initially characterized long-term effects of fluoxetine in cortical, amygdaloid, and hippocampal

brain regions, fluoxetine-induced changes in the expression of BAX and Bcl-2 were opposite to those observed in CUS group, and similar results were observed in bromelain-treated group also. BAX is a pro-apoptotic protein present predominantly in the cytosol [34–36], during the induction of apoptosis, it shifts to mitochondrial membranes. The translocation of BAX to the mitochondria induces cytochrome c release that can trigger apoptosis [37]. Bcl-2 is an anti-apoptotic protein present in the mitochondria and functions as a repressor of apoptosis; overexpression of Bcl-2 protects cells from apoptosis [38]. Hence, it is clear from our study that Bcl-2 and Bax exert a significant effect on neurodegeneration and depression. Also, bromelain treatment could efficiently protect the animals from CUS-induced neurodegeneration. Lastly, the BDNF/TrkB signalling pathway is discussed, which is the most important molecular mechanism involved in depression. Depression can induce a reduction in BDNF expression level, which results in atrophy, neuronal death, and reduced neuronal activity. BDNF/TrkB signalling activates the downstream AKT and ERK signalling. When there is a reduced BDNF level, there is an alteration in the AKT and ERK signalling. Thus, such alterations cause an impairment of CREB signalling, resulting in BDNF downregulation [39]. There are substantial evidences that have proved that the regulation of neuronal plasticity and neurogenesis depends on nerve growth factors like brain-derived neurotrophic factor (BDNF). Many researches indicate that depression and the depletion of neurotrophic support in brain regions like the hippocampus are enhanced by efficacious antidepressant medications. It is believed that BDNF affects the growth and survival of neurons by activating the TrKB. Research on both humans and animals shows that pain and stress are linked to a decrease in BDNF levels and that this loss of neurotrophic support is a factor that causes the atrophic structural alterations in the hippocampus. In the current study, bromelain treatment induces an increase in BDNF and TrkB concentration similar to that of fluoxetine treatment. This increase in BDNF binds to the TrkB receptor and leads to phosphorylation of TrkB and activates downstream cascade which activates AKT and ERK signalling. This ultimately resulted in the improved neurogenesis and neuronal plasticity, which must have prevented neurodegeneration and depression. Rakesh et al. investigated the effect of bromelain in treating Alzheimer's disease in mouse model. The results of that study showed decreased TNF-α and increased BDNF levels, which is similar to our results indicating the activation of neurogenesis and neuronal plasticity, which may prevent neurodegeneration and depression [14].

In another study, it has been reported that BDNF can increase serotonin by activating TrkB, and the expression of serotonin increases after the expression of BDNF and TrkB is increased. Hence, serotonin, BDNF, and TrkB can promote each other [40]. Therefore, bromelain can also regulate the serotonin system by upregulating the expression of BDNF and TrkB in depressive rats, and hence, BDNF and TrkB can further alleviate the symptoms of depressive rats.

5 Conclusion

Findings from the present study show that CUS-induced depression resulted in various alterations in the brain regions resulting in increased levels of inflammatory cytokines, apoptotic proteins and reduced neurotrophic factor, which might have led to neurodegeneration and depressive-like behaviour. Reduced expression of BDNF and TrkB might be the possible cause for neurodegenerative changes in hippocampus and prefrontal cortex. As hypothesized earlier, bromelain has successfully counteracted the neurodegenerative changes in hippocampus and prefrontal cortex. Also, administration of bromelain has effectively attenuated depressive symptoms and related impairments. Hence, the idea that bromelain can be a successful antidepressant medication is supported by the current research. Changes in BDNF levels in the hippocampus and prefrontal cortex may probably be the mediating factor for this impact, indicating a potential role for neurogenesis. Altogether it is suggested that bromelain can be a potent therapeutic agent in combating depression.

Abbreviations

AKT	Serine/threonine kinase 1 or Protein kinase B, PKB
BAX	Bcl-2-Associated X-protein
Bcl-2	B cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
CREB	CAMP response element-binding protein
CUS	Chronic unpredictable stress
ERK	Extracellular signal-regulated kinase
IL-1β	Interleukin-1β (IL-1β)
NFκB	Nuclear factor-κB
TNF-α	Tumour necrosis factor α
TrkB	Tropomyosin receptor kinase B

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Author contributions

All authors have equally contributed to the research work. RP, DJ, and NM carried out the experiment and compiled the data. RP and DJ wrote the manuscript with support from NM and SN. SN supervised the project.

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Availability of data and materials
All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
The experimental protocol was approved by Institutional Animals Ethical Committee, and the experiments were carried out according to the IAEC guidelines (IAEC NO: 02/01/19).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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