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Cryptotanshinone ameliorates cladribine-induced cognitive impairment in rats

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ABSTRACT

Objective: To evaluate the neuroprotective effect of cryptotanshinone against cladribine-induced cognitive impairment in rats.

Methods: Rats were administered with cladribine (1 mg/kg, *p.o.*) and cryptotanshinone (10 and 20 mg/kg, *i.p.*) for four weeks. Behavioral tests such as Morris water maze and elevated plus maze were conducted to check memory impairment caused by cladribine. On day 29, all rats were sacrificed, and the brains were separated for estimation of neuroinflammatory factors, biochemical parameters, neurotransmitters, $A\beta_{(1-42)}$, blood-brain barrier permeability, nuclear factor erythroid 2-related factor 2 (Nrf2), and brain-derived neurotrophic factor (BDNF).

Results: Treatment with cryptotanshinone dose-dependently enhanced spatial memory, improved the levels of neurotransmitter and antioxidant enzymes, and suppressed proinflammatory cytokine release. Cryptotanshinone also decreased $A\beta_{(1-42)}$ accumulation and increased the levels of Nrf2 and BDNF in the hippocampus. Additionally, the histopathological results showed that cryptotanshinone reduced cladribine-induced neuronal death in the hippocampus.

Conclusions: Cryptotanshinone exhibits a promising neuroprotective effect against cladribine-induced cognitive impairment in preclinical studies, and may be a potential phytochemical for the treatment and management of cognitive impairment.

KEYWORDS: Alzheimer's disease; Cladribine; Cryptotanshinone; Neurotransmitters; Neuroprotective; Proinflammatory cytokines; Cognitive deficit

1. Introduction

Cladribine (2-CdA) is an adenosine deaminase inhibitor approved to treat leukemia and multiple sclerosis (MS)[1]. This drug has fatal and life-threatening side effects when administered at doses higher than recommended, including immunodeficiency, myelosuppression, and cognitive impairment (CI) which was highlighted in recent reports. The memory impairment may be due to a deficiency of acetylcholine neurotransmitter & brain-derived neurotrophic factor (BDNF)[2]. Further, cladribine is reported to cause CI *via* accumulation in the central nervous system due to crossing the

Significance

Cryptotanshinone is a diterpene extracted from *Salvia miltiorrhiza* root that exhibits neuroprotective properties. In the current study, cryptotanshinone significantly inhibited the production of proinflammatory cytokines and $A\beta_{(1-42)}$ and improved the levels of Nrf2 and neurotrophic factor in the hippocampus. Therefore, cryptotanshinone may have great potential for the treatment of chemotherapeutic drugs-induced cognitive impairment.

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blood-brain barrier (BBB) in patients with malignancies, and increasing amyloidogenic processing of amyloid precursor protein (APP), leading to robustly increased plaque burden[3]. Available *in–vitro* study linked cladribine promotes amyloid beta (Aβ) peptide production and *C*-terminal fragments in cell cultures[4]. In addition, prolonged cladribine treatment increased Aβ plaque load by more than one-fold in an Alzheimer's disease mouse model. The intracellular neurofibrillary tangles, Aβ, and synaptic degeneration all contribute to the production of extracellular senile plaques. These activities occur in the hippocampus, neocortex, and other subcortical areas that are essential for cognitive function[5].

A clinical study has observed that chronic cladribine causes CI in people with hematological cancers and MS. CI increases trouble in remembering, learning, concentrating, or making decisions in cancer patients[6]. Cancer survivors suffering from memory impairment, slow memory processing, language difficulty, and inability to concentrate are commonly attributed to chemotherapy and are named chemobrain[7]. Mitochondrial dysfunction, neuronal oxidative stress, neurotransmitter disbalance, reduction of neurotrophic factors, and Aβ accumulation are the mechanisms of chemotherapy-induced cognitive impairment (CICI)[8]. Unfortunately, no molecule is yet available to treat all these pathological factors because target drug therapy only prevents neurotransmitter degradation and targets oxidative stress[9]. A number of drugs have been employed by various scientists preclinically and clinically, but they get fail due to the toxic effect of the drug peripherally, reduced BBB penetration, and increased first-pass metabolism.

However, there is a need to look for a compound that targets these mechanisms to achieve neuroprotective and neuromodulatory potential. Natural phytoconstituents are promising and safe for preventing neurodegenerative diseases, including CICI[10]. Cryptotanshinone has biological effects, such as antioxidant, anti-apoptotic, anti-inflammatory, neurotrophic factor enhancer, and amyloid plaque activity attenuating properties[11]. Its low molecular weight and ability to cross the BBB make it a potential neurodegenerative disease treatment[12]. Cryptotanshinone improves proteostasis, mitochondrial complexes activities, and adenosine triphosphate production, decreases reactive oxygen species production, downregulates the proinflammatory cytokine pathways, and increases antioxidant enzyme activities [glutathione (GSH), superoxide dismutase (SOD), and catalase][13]. Cryptotanshinone has also been shown to boost neurotrophic factors like BDNF, which plays a major role in learning and memory[14]. Considering the important role of BDNF in learning and memory associated with CICI, the current study was conducted to determine the neuroprotective effects of cryptotanshinone on cladribine-induced Aβ accumulation and reduced BDNF-associated CI in experimental rats by analyzing behavioral, biochemical, neurotrophic factor, neuroinflammatory markers, and neurotransmitters, and performing histopathological analysis.

2. Materials and methods

2.1. Animals

Wistar rats were obtained for research purposes from the Central Animal House at the ISF College of Pharmacy in Moga, Punjab, India. To check the neuroprotective effect of cryptotanshinone following the administration of cladribine, all rodents were randomized into five groups. All behavioral parameters were measured between 9:00 a.m.-6:00 p.m. during the active phase of rodents.

2.2. Chemicals and reagents

Cryptotanshinone & Cladribine were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO), and ELISA Kits were parched from Krishgen Bio. Sys.

2.3. Treatment schedule

Cladribine (1 mg/kg) was administered orally, and cryptotanshinone (10 and 20 mg/kg) was injected intraperitoneally (*i.p.*) for 28 d. All behavioral activities were carried out between days 1-28, and rats were sacrificed on day 29. Supernatant samples were collected to estimate biochemical, neuroinflammatory markers, neurotransmitters, neurotrophic factor, and β-amyloid, and a histopathological examination was also performed (Supplementary Figure 1).

2.4. Behavioral assessment

2.4.1. Elevated plus maze (EPM) test

The EPM test is used to assess spatial learning and memory. This test was conducted by keeping individual rats at the end of the open arm with their backs to the central platform and noting the time taken for rats to travel to either of the enclosed arms on days 1, 7, 14, 21, and 28. A decrease in transfer latency indicated an improvement in memory activity[15].

2.4.2. Locomotor activity

Each rat's spontaneous locomotion was measured with an actophotometer weekly, and the results were expressed as counts per minute[16].

2.4.3. Morris water maze (MWM)

The MWM is normally used to check the learning and spatial memory of rodents as defined by Morris[17]. The acquisition trial was conducted on days 25, 26, 27, and 28.

2.5. Tissue preparation

On the day of the experiment, the hippocampus part of the brain was isolated, homogenized and centrifuged, and separated supernatants were used to estimate neuroinflammatory markers, biochemical parameters, neurotransmitter levels, neurotrophic factor, and β-amyloid.

2.6. Estimation of the levels of proinflammatory cytokines

The proinflammatory cytokine levels were determined using respective assay kits as per the manufacturer's instructions[18].

2.7. Determination of neurotransmitters

2.7.1. Glutamate & GABA estimation

The levels of GABA and glutamate were determined using HPLC-ECD[19]. The mobile phase contains 100 mM disodium hydrogen phosphate, 25 mM EDTA, and 22% methanol. During an experiment, the electrochemical conditions were kept at +0.65 V and the sensitivity range was set at 5 to 50 nA. After stabilization, frozen brain tissues were homogenized using 0.2 M perchloric acid. The prepared solution was centrifuged at 12 000×*g* for 10 min before filtered through 0.22 mm nylon filters. Following filtering, the filtrate was injected into the HPLC sample injector, and the data was analyzed using Breeze™ 2 software.

2.7.2. Acetylcholine estimation

The colorimetric method (choline/acetylcholine test kit acquired from BioVision Inc., California, USA) was used to assess the acetylcholine level in the brain. As per the manufacturer's instructions, acetylcholine concentration was determined using an ELISA diagnostic kit. The acetylcholine content in the samples was evaluated by comparing the optical density of the samples to the standard curve. The neurotransmitter was quantified as ng/mg of tissue sample[20].

2.8. Biochemical parameters

2.8.1. Estimation of lipid peroxidation

The concentration of lipid peroxidation in hippocampus tissue was measured using the Wills method[21]. Test tubes containing 0.5 mL of homogenate and 0.5 mL of Tris HCl were incubated at 37℃ for 2 h. After incubation, 1 mL of 10% trichloroacetic acid was added. Furthermore, the individual sample was centrifuged at 10 000×*g* for 10 min to separate the supernatant. Then 1 mL of the supernatant was mixed with 1 mL of 0.067% trichloroacetic acid, and the tubes were immersed in boiling water for 10 min. The optical density was determined using a Shimadzu spectrophotometer at 532 nm. A standard curve was drawn using 1-10 nM of 1,1,3,3, tetramethoxypropane, and the results were expressed in μmol/mg protein.

2.8.2. Estimation of nitrite level

The nitrite concentration of the brain tissue supernatant was determined using the Griess reagent, as previously described by Green[22]. Briefly, 100 μL of sample or standard (100, 200, 400, 800, or 1 000 g/mL) were added to 400 μL of distilled water. The solution was combined with 500 μL of Griess reagent, containing 5% phosphoric acid, 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride, and 1% sulphanilamide and the mixture was allowed at room temperature for 5 min.

2.8.3. Estimation of GSH

GSH level was estimated according to Ellman's method[23]. The sample was mixed with an equal volume of 4% sulfosalicylic acid at 4℃ and centrifugated at a speed of 1 200×*g* for 15 min. One mL of supernatant was mixed with 2.7 mL of phosphate buffer (0.1 M, pH 8) and 0.2 mL of 5,5′-dithiobis(2-nitrobenzoic acid). GSH level was measured at 412 nm, using a spectrophotometer (UV-Vis 1700, Shimadzu). The GSH content in the supernatant was calculated using a standard curve and represented as μmol/g protein.

2.8.4. Estimation of glutathione peroxidase (GPx) activity

The GPx activity was evaluated using Rotruck's method[24]. The reaction consists of 0.05 M phosphate buffer, 1.4 U of 0.1 mL glutathione reductase, 1.0 mM EDTA, 1.0 mM sodium azide, 0.25 mM H_2O_2, 1.0 mM glutathione, 0.2 mM nicotinamide adenine dinucleotide phosphate (NADPH), and 0.1 mL phenazine methosulfate. The enzyme activity was determined as U/mg protein at the absorbance of 340 nm.

2.8.5. Estimation of SOD activity

The method of Del Maestro *et al.* was used to evaluate SOD activity[25]. The capacity of the enzyme to scavenge superoxide anion radicals (O_2) is required for SOD activity, which decreases the overall rate of pyrogallol autoxidation.

2.8.6. Estimation of catalase activity

Catalase activity was measured by the method of Aebi *et al*[26]. This method consists of 3 mL of 0.05 M phosphate buffer, 0.05 mL phenazine methosulfate, and 0.019 M H_2O_2. The activity of catalase was measured by the amount of H_2O_2 consumed per minute/mg at 240 nm. Catalase activity was expressed as U/mg protein. All tests were carried out in triplicate.

2.8.7. Mitochondrial complex Ⅰ activity

The activity of complex Ⅰ was measured spectrophotometrically

using the method of King and Howard[27]. The rate of nicotinamide adenine dinucleotide hydrogen (NADH) oxidation was used to evaluate complex Ⅰ activity. The reaction mixture was comprised of 6 mM NADH and 10.5 mM cytochrome C in 0.2 M glycylglycine buffer with a pH of 8.5. A sufficient quantity of solubilized mitochondrial samples was added to initiate the catalytic reaction[27].

2.8.8. Mitochondrial complex Ⅳ activity

The cytochrome oxidase activity in the brain was measured using the method of Sottocasa *et al*[28]. The test mixture was comprised of cytochrome C reduced in 75 mM phosphate buffer. The process was initiated by adding a stabilized mitochondrial sample, and the absorbance at 350 nm was measured.

2.9. Estimation of p-Tau, BACE1, BDNF, Bcl-2, caspase-3 and Nrf2 levels

The levels of caspase-3, Bcl-2, BACE1, p-τ, BDNF, and Nrf2 were determined using ELISA kits and estimated using respective assay kits as per the manufacturer's instructions[29].

2.10. Estimation of amyloid beta$_{1-42}$ (A$\beta_{(1-42)}$)

An A$\beta_{(1-42)}$ ELISA kit was used to determine the A$\beta_{(1-42)}$ concentration[30].

2.11. Detection of osmotic BBB disruption

To test BBB permeability, rats were injected with Evans blue dye. After 30 min, a 300 mL saline perfusion was administered transcardially into the brain. The homogenate sample was centrifuged, and the supernatant was used to determine the dye concentration and expressed as μg/g (brain weight)[31].

2.12. Histopathological analysis

Rats' brains were immediately fixed in 5% formaldehyde, embedded in liquid paraffin wax, sectioned, and stained with hematoxylin and eosin according to the procedure described by Patel and Singh[32]. A fluorescent microscope (102M, Motic Microscopes, China) at 40× magnification was used to examine the stained slices.

2.13. Ethical statement

The Institutional Animal Ethics Committee reviewed and approved the experimental protocol (ISFCP/IAEC/CPCSEA/Meeting No: 28/2020/Protocol No. 477) and experiments were performed in compliance with the guidelines of the Indian National Science Academy.

2.14. Statistical analysis

The mean and standard deviation were calculated using GraphPad Prism 8.0. The significance level of the behavioral results was determined using a two-way ANOVA followed by Bonferroni's *post hoc* test, whereas the biochemical, neurotransmitter, and neuroinflammatory results were determined using a one-way ANOVA followed by Tukey's *post hoc* test. $P<0.05$ was considered statistically significant.

3. Results

3.1. Effects of cryptotanshinone on behavioral activities in cladribine-induced spatial and learning memory deficits in rats

A behavioral test related to learning and memory impairments was performed before and after cladribine and cryptotanshinone treatment. The spatial learning of rats was tested using the MWM, EPM, and actophotometer apparatus. Before treatment with cladribine and cryptotanshinone, no significant change was observed in the behavioral activities of rats. In the EPM, rats administered with cladribine increased the transfer latency compared to the rats in the normal group ($P<0.05$). Interestingly, treatment with cryptotanshinone diminished the transfer latency as compared to the cladribine-challenged rats in a dose-dependent manner ($P<0.05$) (Figure 1). Cladribine also induced an increase in escape latency ($P<0.05$). However, after four weeks of cryptotanshinone treatment, specially at 20 mg/kg, escape latency was significantly decreased ($P<0.05$) (Figure 2). In addition, spontaneous activity in the cladribine group was reduced as compared to the normal group ($P<0.05$), and cryptotanshinone improved the locomotor activity of the rat in a dose-dependent manner ($P<0.05$) (Figure 3).

3.2. Effects of cryptotanshinone on MDA, nitrite, Nrf2, BDNF, p-Tau, BACE1, caspase-3, GSH, GPx, Bcl-2, catalase and SOD in cladribine-administered rats

MDA, nitrite, p-Tau, caspase-3, and BACE1 levels were significantly elevated, while the levels of SOD, GSH, catalase, Nrf2, BDNF, Bcl-2, and GPx were significantly decreased in cladribine-administrated rats compared with the normal rats ($P<0.05$). Cryptotanshinone (10 and 20 mg/kg) substantially diminished the levels of nitrite, MDA, p-Tau, caspase-3, BACE1, and enhanced the levels of GSH, GPx, Nrf2, BDNF, Bcl-2, catalase, and SOD ($P<0.05$) (Tables 1 and 2).

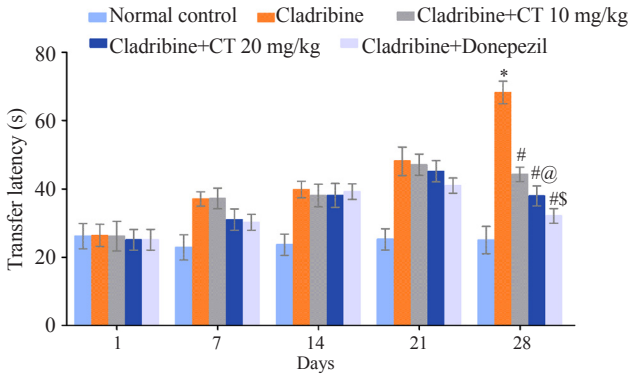

Figure 1. Effect of cryptotanshinone on the transfer latency by elevated plus maze in cladribine-challenged rats. Data are presented as mean ± SD and analyzed by two-way ANOVA followed by Bonferroni's multiple comparison. $^{*}P<0.05$ *vs*. normal control, $^{\#}P<0.05$ *vs*. cladribine 1 mg/kg, $^{@}P<0.05$ *vs*. cryptotanshinone 10 mg/kg, $^{\$}P<0.05$ *vs*. cryptotanshinone 20 mg/kg. CT: cryptotanshinone.

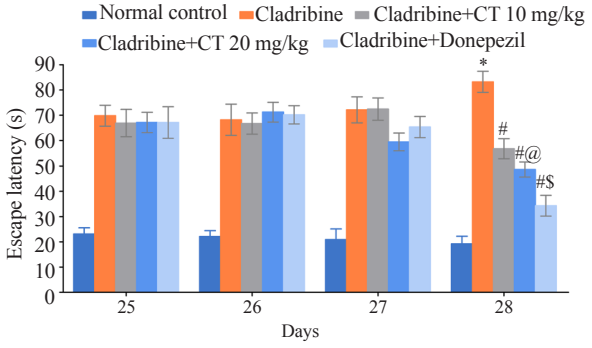

Figure 2. Effect of cryptotanshinone on the escape latency time by Morris water maze in cladribine-challenged rats. Data are presented as mean ± SD and analyzed by two-way ANOVA followed by Bonferroni's multiple comparison. $^{*}P<0.05$ *vs*. normal control, $^{\#}P<0.05$ *vs*. cladribine 1 mg/kg, $^{@}P<0.05$ *vs*. cryptotanshinone 10 mg/kg, $^{\$}P<0.05$ *vs*. cryptotanshinone 20 mg/kg.

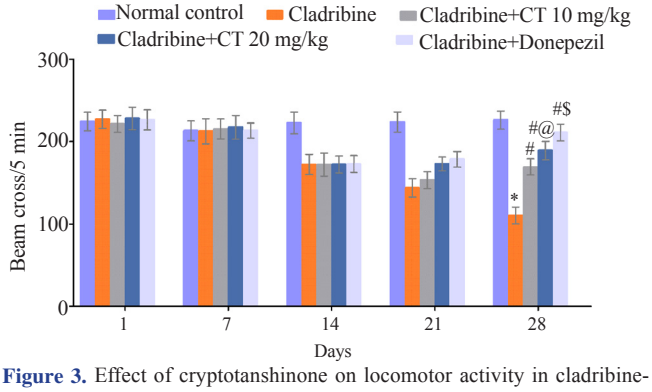

Figure 3. Effect of cryptotanshinone on locomotor activity in cladribine-challenged rats. Data are presented as mean ± SD and analyzed by two-way ANOVA followed by Bonferroni's multiple comparison. $^{*}P<0.05$ *vs*. normal control, $^{\#}P<0.05$ *vs*. cladribine 1 mg/kg, $^{@}P<0.05$ *vs*. cryptotanshinone 10 mg/kg, $^{\$}P<0.05$ *vs*. cryptotanshinone 20 mg/kg.

3.3. Effect of cryptotanshinone on proinflammatory levels in cladribine-administered rats

Acute inflammation plays an important role in the pathogenic progression of neurological disorders; therefore, we checked the modulatory effect of cryptotanshinone on the levels of the proinflammatory cytokine. Our result showed that cladribine-administrated rats showed increased secretion of proinflammatory markers compared with the normal group ($P<0.05$). Treatment with cryptotanshinone markedly reduced the levels of proinflammatory cytokines compared with the cladribine-treated group, especially at a higher dose ($P<0.05$) (Table 2).

3.4. Effect of cryptotanshinone on $A\beta_{(1-42)}$ level in cladribine-administered rats

Cladribine-administrated rats showed a considerable rise in the amount of $A\beta_{(1-42)}$ when compared with the normal group ($P<0.05$). Cryptotanshinone at doses of 10 and 20 mg/kg reduced $A\beta_{(1-42)}$ levels as compared to the cladribine-administrated group in a dose-dependent way ($P<0.05$) (Table 1).

3.5. Effect of cryptotanshinone on mitochondrial complexes in rats administered with cladribine

Rats administrated with cladribine significantly reduced mitochondrial complexes activities as compared to the normal group ($P<0.05$). However, cryptotanshinone significantly increased mitochondrial complexes activities compared to the cladribine group in a dose-dependent way ($P<0.05$) (Table 1).

3.6. Effect of cryptotanshinone on acetylcholine, GABA, and glutamate levels in rats administered with cladribine

As shown in Table 3, cladribine administration reduced the levels of acetylcholine and GABA while elevating glutamate levels compared with the normal group ($P<0.05$). In comparison to the cladribine-treated group, cryptotanshinone significantly enhanced the levels of acetylcholine and GABA and decreased glutamate levels ($P<0.05$). In addition, 20 mg/kg cryptotanshinone was considerably more effective than 10 mg/kg (Table 3).

3.7. Effect of cryptotanshinone treatment on BBB in cladribine-treated rats

Cladribine treatment resulted in BBB integrity breakdown and higher Evans blue dye concentration in the hippocampus compared to the normal control group ($P<0.05$). However, cryptotanshinone improved BBB permeability in a dose-dependent manner as

Table 1. Effects of cryptotanshinone on mitochondrial complexes and cellular marker activities in rats with cladribine-induced cognitive impairment.

Parameters	Normal control	Cladribine	Cladribine + CT (10 mg/kg)	Cladribine + CT (20 mg/kg)	Cladribine + Donepezil
Nrf2 (nM/mg protein)	8.76 ± 0.34	1.72 ± 0.32*	3.71 ± 0.15$^{\#}$	5.87 ± 0.30$^{\#@}$	7.35 ± 0.28$^{\#\$}$
BDNF (ng/g tissue)	22.31 ± 1.30	8.31 ± 2.50*	11.78 ± 1.70$^{\#}$	14.76 ± 2.40$^{\#@}$	18.10 ± 1.50$^{\#\$}$
Bcl-2 (ng/g tissue)	41.40 ± 2.39	7.98 ± 1.21*	13.34 ± 2.21$^{\#}$	24.12 ± 1.42$^{\#@}$	35.21 ± 4.05$^{\#\$}$
Caspase-3 (ng/g tissue)	0.13 ± 0.04	15.21 ± 3.02*	11.01 ± 0.98$^{\#}$	7.10 ± 1.09$^{\#@}$	4.21 ±1.01$^{\#\$}$
BACE1 (ng/g tissue)	0.65 ± 0.09	5.13 ± 0.16*	3.32 ± 0.16$^{\#}$	2.67 ± 0.42$^{\#@}$	1.78 ± 0.19$^{\#\$}$
p-Tau (pg/g tissue)	7.13 ± 0.84	27.21 ± 4.12*	21.21 ± 2.21$^{\#}$	13.67 ± 3.11$^{\#@}$	9.34 ± 1.45$^{\#\$}$
Complex- Ⅰ (nM/mg protein)	11.08 ± 0.13	2.87 ± 0.90*	4.89 ± 0.21$^{\#}$	6.98 ± 0.28$^{\#@}$	9.06 ± 0.10$^{\#\$}$
Complex-Ⅳ (nM/mg protein)	298.21 ± 9.10	123.23 ± 7.13*	168.45 ± 8.45$^{\#}$	203.34 ± 6.34$^{\#@}$	232.12 ± 7.21$^{\#\$}$
$A\beta_{(1\text{-}42)}$ (pg/g protein)	1.73 ± 0.71	12.11 ± 1.76*	7.91 ± 2.71$^{\#}$	4.98 ± 1.30$^{\#@}$	3.02 ± 1.42$^{\#\$}$

Data are presented as mean ± SD and analyzed by one-way ANOVA followed by Tukey's *post hoc* test. $^{*}P$<0.05 *vs*. normal control, $^{\#}P$<0.05 *vs*. cladribine 1 mg/kg, $^{@}P$<0.05 *vs*. cryptotanshinone 10 mg/kg, $^{\$}P$<0.05 *vs*. cryptotanshinone 20 mg/kg. CT: cryptotanshinone.

Table 2. Effects of cryptotanshinone on the levels of MDA, nitrite, GSH, GPx, SOD, catalase, TNF-α, IL-1β and IL-6 in rats with cladribine-induced cognitive impairment.

Parameters	Normal control	Cladribine	Cladribine + CT (10 mg/kg)	Cladribine + CT (20 mg/kg)	Cladribine + Donepezil
MDA (μmol/mg protein)	1.41 ± 0.17	6.96 ± 0.17*	4.21 ± 0.12$^{\#}$	3.34 ± 0.10$^{\#@}$	2.21 ± 0.22$^{\#\$}$
Nitrite (μg/mL)	102.90 ± 8.08	245.21 ±12.32*	112.40 ± 12.22$^{\#}$	135.20 ± 8.20$^{\#@}$	116.20 ± 8.03$^{\#\$}$
GSH (μmol/g protein)	0.090 ± 0.010	0.007 ± 0.001*	0.020 ± 0.003$^{\#}$	0.040 ± 0.001$^{\#@}$	0.070 ± 0.003$^{\#\$}$
GPx (U/mg protein)	16.32 ± 2.32	4.81 ± 0.29*	8.23 ± 0.19$^{\#}$	11.21 ± 0.62$^{\#@}$	13.87 ± 0.59$^{\#\$}$
SOD (U/mg protein)	6.73 ± 0.71	1.09 ± 0.23*	2.21 ± 0.21$^{\#}$	3.43 ± 0.41$^{\#@}$	5.01 ± 0.87$^{\#\$}$
Catalase (U/mg protein)	7.21 ± 0.19	1.52 ± 0.29*	2.87 ± 0.29$^{\#}$	3.89 ± 0.18$^{\#@}$	5.21 ± 0.87$^{\#\$}$
TNF-α (pg/mg protein)	52.21 ± 4.21	160.23 ± 7.42*	110.21 ± 5.16$^{\#}$	85.41 ± 4.55$^{\#@}$	66.1 ± 5.12$^{\#\$}$
IL-1β (pg/mg protein)	44.20 ± 3.76	197.21 ± 7.21*	141.12 ± 8.09$^{\#}$	110.15 ± 7.21$^{\#@}$	91.21 ± 3.06$^{\#\$}$
IL-6 (pg/mg protein)	30.98 ± 3.98	141.31 ± 6.12*	109.11 ± 6.87$^{\#}$	82.18 ± 5.96$^{\#@}$	58.21 ± 6.92$^{\#\$}$

Data are presented as mean ± SD and analyzed by one-way ANOVA followed by Tukey's *post hoc* test. $^{*}P$<0.05 *vs*. normal control, $^{\#}P$<0.05 *vs*. cladribine 1 mg/kg, $^{@}P$<0.05 *vs*. cryptotanshinone 10 mg/kg, $^{\$}P$<0.05 *vs*. cryptotanshinone 20 mg/kg.

Table 3. Effects of cryptotanshinone on the level of neurotransmitters in rats with cladribine-induced cognitive impairment (ng/mg tissue).

Parameters	Normal control	Cladribine	Cladribine + CT (10 mg/kg)	Cladribine + CT (20 mg/kg)	Cladribine + Donepezil
GABA	48.20 ± 2.89	12.32 ± 1.19*	23.76 ± 1.26$^{\#}$	29.49 ± 2.65$^{\#@}$	38.34 ± 2.17$^{\#\$}$
Glutamate	53.09 ± 2.54	109.91 ± 6.87*	88.65 ± 5.18$^{\#}$	71.11 ± 4.89$^{\#@}$	62.27 ± 3.78$^{\#\$}$
Acetylcholine	6.21 ± 1.09	1.23 ± 0.11*	2.91 ± 0.12$^{\#}$	4.03 ± 0.27$^{\#@}$	4.99 ± 0.67$^{\#\$}$

Data are presented as mean ± SD and analyzed by one-way ANOVA followed by Tukey's *post hoc* test. $^{*}P$<0.05 *vs*. normal control, $^{\#}P$<0.05 *vs*. cladribine 1 mg/kg, $^{@}P$<0.05 *vs*. cryptotanshinone 10 mg/kg, $^{\$}P$<0.05 *vs*. cryptotanshinone 20 mg/kg.

compared to the cladribine group by retaining BBB integrity (P<0.05) (Figure 4).

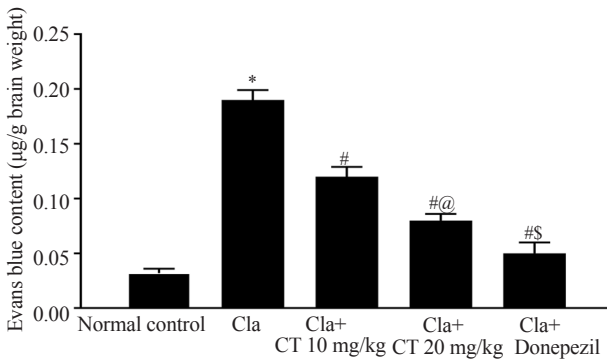

Figure 4. Effect of cryptotanshinone on Evans blue content in the brains of cladribine-challenged rats. Data are presented as mean ± SD and analyzed by one-way ANOVA followed by Tukey's *post hoc* test. $^{*}P$<0.05 *vs*. normal control, $^{\#}P$<0.05 *vs*. cladribine 1 mg/kg, $^{@}P$<0.05 *vs*. cryptotanshinone 10 mg/kg, $^{\$}P$<0.05 *vs*. cryptotanshinone 20 mg/kg. Cla: cladribine.

3.8. Effect of cryptotanshinone on histopathological alteration in cladribine–treated rats

In the control group, hematoxylin and eosin staining of hippocampus tissues revealed normal neurons with intact nuclear membranes and cell membrane integrity. Neuronal apoptosis was found in the cladribine group, resulting in a considerable drop in the number of neurons as compared to the normal control group (P<0.05). However, the cryptotanshinone and donepezil groups had fewer neurodegenerative hippocampus changes than the cladribine group. Furthermore, treatment with a high dose of cryptotanshinone decreased neurodegeneration in the hippocampus more significantly than a low dose of cryptotanshinone (P<0.05) (Figure 5).

4. Discussion

In the current work, we evaluated the neuroprotective impact of cryptotanshinone against cladribine-induced learning and memory deficits in rats using behavioral, biochemical, inflammatory, and

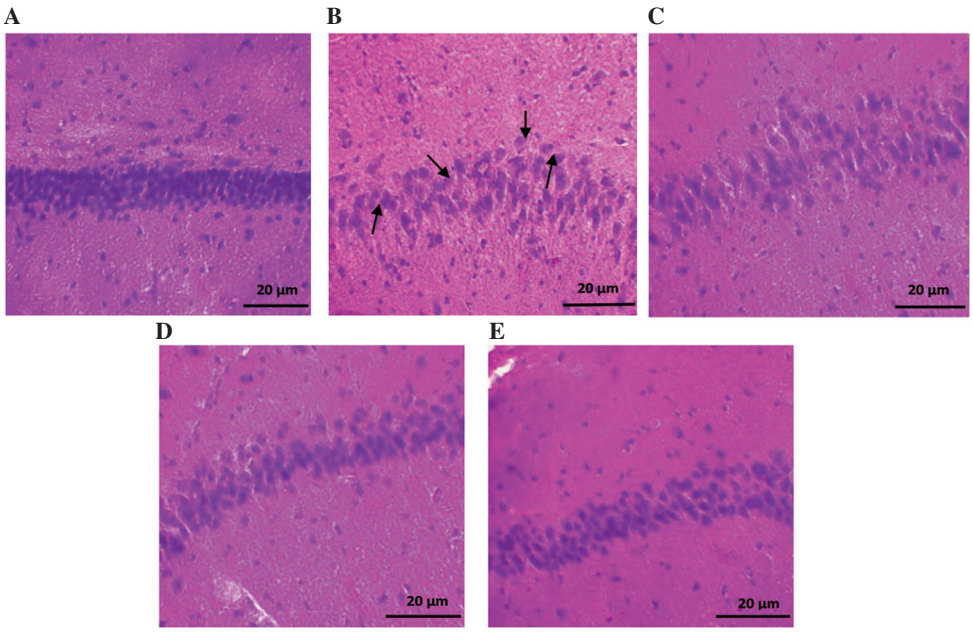

Figure 5. Effect of cryptotanshinone on histopathological changes in the brains of cladribine-challenged rats (Magnification 40×, scale bar: 20 μm). (A) Fluorescent microphotographs of hematoxylin and eosin stained sections of the hippocampus region show normal neurons in the normal group. (B) Cladribine administered rats show apoptotic (black arrow) nuclei in the hippocampus. (C & D) Rats treated with cryptotanshinone at doses of 10 mg/kg and 20 mg/kg show prominently improved neuronal cells and reduced apoptotic cells. (E) Rats treated with donepezil show significant regeneration of hippocampal neuronal cells.

neurochemical analyses. Clinical studies have shown that hairy-cell leukemia, lymphoid cancer, and MS patients who took chronic cladribine chemotherapy showed CI, including troubles in remembering, learning, concentrating, making decisions, and attention, compared to those who did not receive chemotherapy treatment[33]. Previous research using mouse models showed that cladribine-induced memory impairment is related to increased Aβ plaque load[4]. In the current investigation, we observed that rats administered cladribine had behavioral and biochemical abnormalities, lower neurotransmitter concentrations, and produced proinflammatory cytokines in the hippocampus. Cladribine administration for 28 d impaired memory activity confirmed by behavioral tests. An alteration in memory was confirmed by the MWM test, as evidenced by increased latency time. Similarly, a previous study found that chronic cladribine administration increased the latency in T maze learning tasks[4]. Whereas cryptotanshinone treatment significantly attenuated cladribine-induced memory deficits, which is consistent with previous findings. These results strongly suggested that cryptotanshinone exerts neuroprotective effects dose-dependently by alleviating memory and learning deficits induced by chronic chemotherapy[34].

Oxidative stress is essential for activating and generating a variety of cell signaling pathways that result in the formation of the toxic substance, which promotes neurological diseases[35]. It has been shown that hippocampal neurons are very vulnerable to oxidative stress, which may be caused by generation of free radicals and the neuronal cell's capacity to defend against them[36]. Antioxidants from natural sources play a vital role in delaying the onset as well as reducing the progression of neurological diseases. Therefore, we explored the underlying mechanism of cryptotanshinone, and the levels of antioxidative enzymes and neurotrophic factors were measured. It has long been established that LPO level is elevated in the Alzheimer's disease brain as well as in the postmortem brain of subjects with CI[37,38]. Nrf2 regulates antioxidant enzymes (SOD, catalase, GSH, and GPx) in a number of tissues, including neural cells[39]. In our study, the activities of SOD, GSH, GPx, catalase, and Nrf2 were markedly decreased in the cladribine group, accompanied by an increase in the level of MDA, suggesting that oxidative stress injury was initiated in the hippocampal tissues of rats with chemotherapy-induced CI. Our results are compatible with a number of investigations demonstrating the antioxidant effect of cryptotanshinone in rodents with different neurological disorders [CT and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models][40]. Therefore, the protective effect of cryptotanshinone against cladribine-induced learning and memory impairments could be attributable to enhanced antioxidant enzyme activities.

Research has identified BDNF plays an important role in learning and memory function. In the current study, the beneficial effects of cryptotanshinone on learning and memory have been associated with increased BDNF levels in the hippocampus, which has also been observed using different animal models. The learning and memory-improving effects of cryptotanshinone may be due to an increase in

the level of acetylcholine[41]. According to a report, BDNF promotes the release of acetylcholine from hippocampal synaptosomes containing the terminals of septal cholinergic neurons. The reciprocal regulation of acetylcholine and BDNF in the hippocampus indicates that neurotrophins may affect synaptic plasticity and memory. The cladribine-administered group had lower GABA and acetylcholine, as well as higher glutamate levels compared to the normal group. However, cryptotanshinone therapy dose-dependently prevented cholinergic neuronal death and balanced GABA and glutamate levels.

Proinflammatory markers play a significant role in inflammation and are related to an increased risk of dementia[42]. In addition, these elevated levels of proinflammatory mediators caused the death of hippocampal neurons and the progression of neurological diseases. In the current study, cladribine stimulated the production of proinflammatory markers and initiated inflammatory cascades, resulting in decreased neuronal function. However, the levels of proinflammatory markers were significantly diminished by treatment with cryptotanshinone. A previous study also reported that cryptotanshinone significantly downregulated the proinflammatory level in BV-2 microglial cells induced by LPS in a dose-dependent manner[43].

The BBB regulates the influx and efflux of biological molecules that are important for the neuronal function of the brain. Evans blue dye is the most widely used indicator in *in–vivo* studies to determine the integrity of the BBB. In the current study, Evans blue dye was used to evaluate BBB deterioration induced by cladribine. The current research showed that BBB membrane breakdown in the brains of cladribine-challenged rats caused an elevated Evans blue concentration. Previous research has also demonstrated that cladribine has a high affinity toward the brain and easily crosses the BBB. Similarly, our results suggest that cladribine may alter the permeability of the BBB, triggering a destructive effect on the central nervous system and hippocampal neuronal death. However, cryptotanshinone improved BBB permeability due to its neuroprotective properties in a dose-dependent manner.

Increased levels of Aβ in dementia result in the formation of senile plaques containing Aβ depositions. Our current results agreed with previous findings. After treatment with cryptotanshinone, $A\beta_{42}$ aggregation was effectively inhibited and cellular apoptosis was reduced. This study suggests that cryptotanshinone may be beneficial for inhibiting or preventing the progression of Alzheimer's disease due to reduced $A\beta_{42}$ aggregation. Nrf2 is required for the regulation of cellular redox homeostasis and neuroinflammation. According to the research, decreasing BACE1 expression *via* Nrf2 activation alleviates cognitive deficits and Aβ buildup. Furthermore, it was shown that cryptotanshinone has neuroprotective properties by activating the Nrf2 pathway.

In conclusion, cryptotanshinone can be explored as a potential neuroprotective agent against cladribine-induced cognitive impairment in rats. Although the study mainly focused on the neuroprotective effects of cryptotanshinone on cognitive impairment, it is important to note that the cladribine caused more mortality of rats as compared to other standard models. Therefore, it is imperative to utilize an adequate number of animals in this particular model. Additionally, we examined only a few parameters to evaluate the neuroprotective effects of cryptotanshinone, but in the future, researchers could conduct further measurements of different molecular parameters using Western blotting or real time PCR analysis. Furthermore, the addition of a drug parse group may add valuable information, which is lacking in our protocol. In future, further study is required to investigate possible pathways through which cladribine induces AD and estimate the neuroprotective effects of cryptotanshinone for longer durations to confirm more anti-Alzheimer effects of these molecules in various AD animal models before starting the clinical study.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Authors' contributions

KRA conducted the experiment, collected data, and wrote the manuscript. GDG and SS designed, reviewed, analyzed and edited manuscript.

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