Antiepileptic Activity of *Callicarpa kwangtungensis* Chun Extract in Pilocarpine Induced Epilepsy in Mice *via* Modulating GABAergic and DAergic Transmission

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The effect of *Callicarpa kwangtungensis* Chun (CKC) was evaluated in Pilocarpine (P350, 350 mg/kg)-induced epilepsy in mice. The behavioral patterns of experimental mice were investigated by the number of convulsed animals, survivors, after P350-induced convulsions, latency to 1st convulsion, and latency to death. The administration of CNC extended both the latency to the first convulsion and the time to death in a dose-dependent manner. The role of receptors in the anti-convulsive action of CNC was investigated using a combination of atropine (cholinergic receptor antagonist), memantine (NMDA-type glutamate receptors blocker), and diazepam (GABA agonist). The CNC increased latency to the 1st convulsion and to death in diazepam combination while there was no significant alteration in other two combination, suggesting that CNC anticonvulsive action is mediated through the GABA receptor. The CNC increased the striatal concentration of dopamine (DA) and DOPAC in the brain, suggesting that it prevents convulsion in animals by inhibiting DA metabolism. The CNC improved the antioxidant status of brain as evidenced by decrease in level of TABARS, carbonyl protein, and nitric oxide, whereas the levels of sulfhydryl protein, SOD, and CAT were increased. Thus, the anti-epileptic potential of *Callicarpa kwangtungensis* Chun is accomplished by modulating GABAergic and DAergic transmission.

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INTRODUCTION

Globally, epilepsy is the third most common neurological condition, after stroke and Alzheimer's (Beghi 2007). The prevalence of epilepsy has steadily increased in recent decades, a trend that has been ascribed to a number of variables, such as enhanced diagnostic skills, longer life expectancies, and genetic and environmental predispositions. The increasing incidence presents considerable problems to global healthcare systems, exacerbating the socioeconomic burden related to its management (Ioannou *et al.* 2022).

Research suggests that a significant percentage of epilepsy patients may have spontaneous remission; however, the mechanisms involved are not well comprehended (Fisher *et al.* 2005). Despite progress in treatment methods, attaining perfect seizure control continues to be hard for numerous individuals. Antiepileptic medicines (AEDs) frequently serve as the primary treatment option; however, they include intrinsic limitations. Although

certain patients exhibit positive responses to antiepileptic drugs (AEDs), a significant proportion develops drug-resistant epilepsy, which is characterized by the inability to attain prolonged seizure independence despite undergoing trials of a minimum of two well-tolerated and suitably selected pharmacological treatments (Turski *et al.* 1989; Howard *et al.* 2011; Catterall 2014). This medication resistance highlights the pressing necessity for innovative therapeutic strategies. The side effects of present antiepileptic drugs, such as cognitive impairment, hepatotoxicity, and teratogenicity, often undermine patient adherence and quality of life, highlighting the urgent need for safer and more effective treatments.

In the pursuit of novel medicines, natural compounds have surfaced as a viable pathway. Historically, natural compounds have proven fundamental to drug discovery, providing a wide array of bioactive molecules with various pharmacological effects (Butler *et al.* 2014). Their function in alleviating neurological disorders is especially significant, as numerous natural compounds demonstrate neuroprotective, anti-inflammatory, and antioxidative characteristics. These chemicals possess the capacity to influence disease progression and serve as a foundation for the development of medicines with innovative modes of action (Cragg *et al.* 1997; Luo *et al.* 2014).

The investigation of natural compounds for anticonvulsant properties has accelerated, with numerous studies validating their effectiveness in preclinical epileptic models (Hassanzadeh *et al.* 2021; Sharifi-Rad *et al.* 2021). Compounds sourced from plants, fungi, and marine organisms have demonstrated potential in targeting pathways associated with seizure development and propagation (Copmans *et al.* 2019; He *et al.* 2024; Wang *et al.* 2024). Flavonoids, terpenoids, and alkaloids have demonstrated modulatory effects on neurotransmitter systems, ion channels, and oxidative stress, which are critical factors in epileptogenesis (Sharifi-Rad *et al.* 2021). These findings underscore the potential of natural products as a source of lead compounds for the advancement of next-generation anticonvulsants.

Thus, epilepsy presents considerable medical and socioeconomic challenges, the incorporation of natural product research into medication discovery offers enormous potential. By utilizing the therapeutic potential of these bioactive molecules, researchers may discover novel techniques to meet the unmet requirements of epilepsy patients, especially those with drug-resistant variants of the condition (de Boer *et al.* 2008).

The Verbenaceae family includes the genus *Callicarpa*, which is home to about 190 species mainly found in the tropics and subtropics of Asia and Oceania (Zhang *et al.* 2023). This genus has long been acknowledged for its therapeutic properties, with numerous species being employed in diverse cultural therapies. *Callicarpa macrophylla*, *Callicarpa nudiflora*, and *Callicarpa dichotoma* are notable for their historical uses in traditional medicine, treating a wide range of diseases (Soni *et al.* 2014). These herbs have been essential to traditional medicinal methods, esteemed for their enduring therapeutic powers over generations (Yang *et al.* 2021; Kim *et al.* 2022; Ma *et al.* 2022).

Comprehensive phytochemical studies of the *Callicarpa genus* have uncovered a substantial array of bioactive chemicals. Prominent among them are terpenoids, especially diterpenoids, in addition to phenylethanoids, phenylpropanoids, and flavonoids. These chemical compounds are linked to various pharmacological actions. Research underscores their potential as anti-inflammatory agents and their capacity to facilitate hemostasis.

Furthermore, chemicals from this genus exhibit antitubercular, antiplatelet aggregation, and cytotoxic properties, as well as the ability to stimulate neurite outgrowth, highlighting their significance in both traditional and contemporary medicine (Zhao *et al.* 2021; Huang *et al.* 2022; Li *et al.* 2022).

Callicarpa kwangtungensis Chun (CNC), a notably important species, is a shrub indigenous to southern mainland China. This plant has a significant role in local folk medicine, with its leaves and twigs employed to address ailments such as reproductive performance (Chen *et al.* 2020), antioxidant activity (Cai *et al.* 2014), and anti-inflammatory activities (Zheng *et al.* 2021). The traditional applications highlight the cultural significance of the *Callicarpa genus* and indicate its potential for future pharmaceutical research and development. However, no study has reported the antiepileptic potential of *Callicarpa kwangtungensis* Chun. Thus, concerning the above, in the present study, the aim was to explore the pharmacological activity of CKC against Pilocarpine (PILO) induced epilepsy in mice.

The pilocarpine-induced epilepsy model possesses considerable pharmacological significance due to its accurate representation of human temporal lobe epilepsy (TLE). Pilocarpine, a muscarinic cholinergic receptor agonist, precipitates status epilepticus by facilitating excessive neuronal excitation, ultimately resulting in chronic epileptogenesis. This procedure emulates essential pathophysiological characteristics of natural temporal lobe epilepsy, such as hippocampal sclerosis, mossy fiber sprouting, and gliosis, rendering it an indispensable instrument in seizure research and antiepileptic medication development (Turski *et al.* 1983; Cavalheiro *et al.* 1987).

Evidence indicates that, when meticulously controlled, pilocarpine-induced seizures effectively replicate spontaneous seizure occurrences in relation to experimental bias. Electrophysiological recordings and behavioral analysis demonstrate that these seizures exhibit comparable features and temporal profiles to normally occurring seizures (Mazarati *et al.* 1998). Despite pilocarpine's action through a particular cholinergic route, the ensuing neuroplastic alterations resemble those seen in idiopathic temporal lobe epilepsy, indicating minimal pharmacological bias in the induction process. This model continues to be a highly dependable experimental framework for assessing possible anticonvulsant treatments. These data substantiate the efficacy of pilocarpine in simulating clinically pertinent epileptogenesis without considerable methodological bias, hence enhancing our therapeutic understanding.

EXPERIMENTAL

Chemicals

All the chemicals were procured from Sigma Aldrich USA, unless otherwise stated.

Collection and Identification of Plant

The *Callicarpa kwangtungensis* Chun plant was collected at peak flowering to early fruiting stage from the botanical garden of the People's Hospital of Nanchang and was identified by an experienced botanist. This growth stage was selected based on standard phytochemical collection practices, as the plant is metabolically most active during this period, ensuring a higher concentration of bioactive secondary metabolites such as flavonoids, terpenoids, and phenolic compounds. A sample specimen voucher was stored in the herbarium of the Institute (HB/2024/021).

Preparation of CNC Extract

The whole plant material was collected and thoroughly cleaned with tap water to remove soil particles, dust, and other extraneous matter. The cleaned plant material was initially air-dried at room temperature for 24 h to eliminate surface moisture. Subsequently, the material was shade-dried in a well-ventilated area at ambient temperature (25 to 35 °C) for approximately two weeks, following standard phytochemical preparation protocols. Since the plant material included fruits, which inherently retain higher moisture content compared to leaves and stems, the adequacy of the drying period was closely monitored. The drying progress was assessed through regular physical inspection, ensuring that the leaves and stems became brittle while the fruits exhibited hardness and shrivelling without residual softness. Additionally, representative samples were weighed at intervals of two to three days, and drying was considered complete when no further reduction in weight was observed over two consecutive measurements. To confirm minimal residual moisture, a subsample was subjected to oven-drying at 105 °C for three hours, and the resulting weight loss was found to be negligible. The completely dried plant material was then milled to a coarse powder using a ball mill and sieved through a 200-mesh sieve to obtain uniform particle size. The powdered material was stored in airtight containers at room temperature until further use.

Preparation of Petroleum Ether Extract

A total of 500 g of the dried, powdered plant material was subjected to exhaustive extraction using a Soxhlet apparatus with petroleum ether (boiling range 40 to 60 °C) for 36 hours. Petroleum ether was selected as the extraction solvent due to its non-polar nature, which facilitates the selective extraction of lipophilic phytoconstituents such as fatty acids, terpenoids, and phytosterols. Its low boiling point further enabled efficient solvent recovery under reduced pressure without degrading thermolabile compounds. The obtained extract was concentrated using a rotary evaporator under reduced pressure and further dried using a vacuum pump to yield 25 g of semi-solid crude extract. The extract was stored in an airtight container at 4 °C until further analysis and bioassay evaluation.

Preliminary Phytochemical Screening

The petroleum ether extract was subjected to qualitative phytochemical screening to identify the major classes of non-polar secondary metabolites, following standard procedures:

Test for phytosterols (Liebermann–Burchard Test)

Two milliliters of the extract solution were mixed with 2 mL of chloroform. To this, 2 to 3 drops of acetic anhydride were added, followed by the careful addition of 1 mL concentrated sulfuric acid along the side of the test tube. The formation of a bluish-green color indicated the presence of phytosterols.

Test for terpenoids (Salkowski's Test)

A small quantity of the extract was dissolved in 2 mL of chloroform. To this, 2 mL of concentrated sulfuric acid was carefully added to form a layer. A reddish-brown coloration at the interface indicated the presence of terpenoids.

Test for fixed oils and fats (spot test)

A small amount of the extract was pressed between two filter papers. The appearance of a persistent, translucent oily spot indicated the presence of fixed oils and fats.

Detection of fats and oils (Sudan III staining)

The extract was dissolved in ethanol, and a few drops of Sudan III solution were added. The formation of red-colored droplets indicated the presence of fats and oils.

Test for alkaloids

The extract was treated with Dragendorff's reagent. The absence of an orange or reddish-brown precipitate indicated the absence of alkaloids.

Test for flavonoids

A small quantity of the extract was treated with a few drops of 10% lead acetate solution. The absence of a yellow precipitate indicated the absence of flavonoids.

Test for tannins

To a small portion of the extract solution, a few drops of 5% ferric chloride solution were added. The absence of a dark blue or greenish-black color indicated the absence of tannins.

Test for saponins (foam test)

A small quantity of the extract was vigorously shaken with distilled water in a test tube. The absence of persistent froth indicated the absence of saponins.

Test for glycosides (Keller–Killiani test)

Two milliliters of the extract solution were mixed with 1 mL of glacial acetic acid containing a trace amount of ferric chloride. This was carefully under-layered with 1 mL of concentrated sulfuric acid. The absence of a reddish-brown layer at the junction indicated the absence of cardiac glycosides.

Animals

Male Swiss mice, weighing 25 to 30 g, were obtained from the Institutional Animal House and housed in a strictly aseptic environment. The mice were maintained under controlled conditions, including a 12-h light/dark cycle at a constant temperature of 21 to 24 °C, with uninterrupted access to food and water. To ensure acclimatization to the laboratory environment and minimize stress, the animals were kept under these conditions for seven days before the commencement of the experiment.

Ethical Statement

All animal experiments were conducted in accordance with international, national, and institutional guidelines for the care and use of laboratory animals. The study protocol was reviewed and approved by the Animal Ethical Committee of The People's Hospital of Nanchang, China (Approval Number: K-ky2022023). All procedures adhered to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize animal suffering and to use the minimum number of animals required to achieve reliable scientific results.

Experimental Protocol

The mice were acutely administered CNC extract at doses of 25, 50, or 100 mg/kg *via* intraperitoneal injection (i.p.). Thirty minutes following the CNC extract administration, the animals received an injection of pilocarpine (PILO) at a dose of 350 mg/kg (i.p.) to induce seizures.

For the combination treatment experiments, additional pharmacological agents were incorporated into the protocol to evaluate potential interactions. Specifically, atropine (ATR, i.p.), diazepam (DZP, i.p.), memantine (MEM, orally), flumazenil (FLUM, i.p.), or nimodipine (NIMO, orally) were administered 15 min prior to the CNC extract injection (i.p.). This timing ensured that these agents were introduced 45 min prior to the PILO injection (i.p.), allowing adequate pharmacodynamic interactions to occur.

The design of this protocol enabled the assessment of both the individual and combined effects of CNC extract and these agents on the PILO-induced seizure model, providing valuable insights into their potential therapeutic or synergistic roles.

Behavioural Testing

Mice were distributed into the following groups:

Group 1: Pilocarpine (PILO: 350 mg/kg, i.p.)

Group 2: CNC+PILO (CNC: 25, 50, 100 mg/kg, i.p.) followed by Pilocarpine administration (350 mg/kg), 30 min later.

The details of combination experiments were as follows (the number given in front of CNC represent dose in mg/kg):

1. ATRP1+CNC50; ATRP10+CNC100;
2. MEM2+CNC100
3. DZP0.2+CNC50; DZP0.5+CNC100;

After the PILO injection was administered, the animals exhibited signs of reduced activity (hypoactivity) and specific physical behaviours, including oro-facial motions, profuse salivation, eye squinting, quivering of their vibrissae (whiskers), and yawning, approximately 30 min later. Generalized seizures and indications of limbic status epilepticus were noted 40 to 80 min post-injection. The main characteristics evaluated during this period were the latency to the first convulsion and the total survival duration, recorded up to a maximum of 2 h. Furthermore, the quantity of animals that endured during a 24-h observation period was documented.

In tests examining monoamine and amino acid concentrations, the animals were euthanized by decapitation immediately after their initial convulsion. In the PILO-only group, this generally ensued within 30 min, as subjects in this cohort rarely endured beyond that duration. Conversely, in groups pretreated with CNC prior to PILO administration, animals were killed either 2 h after their initial convulsion or at the 2-h interval if they survived that duration. The selected time points were meticulously aligned with the survival trends identified in each group.

Following euthanasia, the brains of the animals were dissected to extract the striata. The aforementioned brain areas were utilized to create homogenates for the quantification of monoamine and amino acid concentrations. This methodology guaranteed accurate biochemical analysis pertinent to the study's aims.

Monoamine Level Determination

To test dopamine and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), 10% homogenates were prepared using the striata from all of the groups that had either

been treated with PILO or pretreated with CNC. This process was followed, with some minor adjustments, in accordance with a procedure that had been described earlier.

Oxidative Stress Markers in Brain

The effect of CNC on lipid and protein oxidative damage was performed by the estimating the concentration of TBARS, carbonyl protein, nitric oxide levels, sulfhydryl protein, and activity of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) were quantified following previously reported procedures.

Statistical Analysis

The data for latencies to the 1st convulsion and to death were analyzed by the non-parametric Kruskal–Wallis test, followed by the Dunn's as a post hoc multiple comparisons test, and the frequency to death by the Log-rank (Mantel–Cox) test. Two-way ANOVA (figures from GraphPad Prism 5), followed by the Mack–Skillings statistical test (a nonparametric Two-way ANOVA used for incomplete block designs) and by the Dwass–Steel–Critchlow–Fligner test for multiple comparisons were used (XLSTAT), for the association experiments with two or more drugs. All other cases were analyzed with One-way ANOVA, followed by the Student–Newman–Keuls as the test post hoc. The results were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

There have been countless maladies that have been treated with plant-based medicines ever since the beginning of folklore (Saklani and Kutty 2008). There have been a number of studies that have demonstrated their great efficacy against non-infectious diseases as well as infectious diseases, which includes neurological problems (Balunas and Kinghorn 2005). The use of these phytochemicals, which target specific receptors, provides relief from the disease while causing less adverse effects than the standard medicines.

Epilepsy, particularly in cases involving convulsive seizures, has a profound effect on behavioral patterns, resulting in cognitive, emotional, and social difficulties (Engelborghs *et al.* 2000; Fisher *et al.* 2005; Scharfman 2007). Convulsions interfere with neuronal activity, resulting in postictal confusion, aggression, or lethargy. Chronic seizures are associated with anxiety, depression, irritability, and memory impairments. In children, epilepsy frequently presents as hyperactivity or social withdrawal, whereas adults may encounter reduced cognitive flexibility and heightened emotional instability. The unpredictable nature of seizures increases psychological stress and social stigma, adversely impacting quality of life. However, despite of the advances in therapeutic options, still the patients of epilepsy are non-responsive to conventional pharmacotherapy due to poor blood-brain barrier penetrability, and serious side-effects due to long term usage (Moosa 2019; Löscher *et al.* 2020). Thus, it is important to find new and efficacious therapeutic agents. Therefore, in the present study, the goal was to investigate the impact that *Callicarpa kwangtungensis* Chun. (CKC) has on the development of epilepsy in mice that has been caused by Pilocarpine (PILO).

The petroleum ether extraction of the dried whole plant material of CKC yielded 25 g of concentrated crude extract from 500 g of powdered plant material, corresponding to an extraction yield of 5% (w/w). The extract was obtained as a semi-solid, dark green to brownish mass with an oily texture and characteristic odor, typical of non-polar

phytochemical-rich fractions. Preliminary phytochemical screening revealed the presence of phytosterols, terpenoids, and fixed oils, while alkaloids, flavonoids, tannins, saponins, and glycosides were absent. These results are consistent with the non-polar nature of petroleum ether, which preferentially extracts lipophilic constituents such as fatty acids, sterols, and terpenoids. The presence of these non-polar bioactive compounds is consistent with previous reports on *Callicarpa* species, which have been shown to possess neuropharmacological activities attributed to their sterols and terpenoids (Li *et al.* 2016; Wang *et al*. 2018). The moderate extraction yield and phytochemical profile obtained in this study support the rationale for selecting petroleum ether as the solvent, as it effectively enriched the extract with bioactive non-polar secondary metabolites. The dried extract was subsequently used for bioassay evaluation to investigate its potential pharmacological properties. Animal models, such as pilocarpine-induced epilepsy in mice, provide a valuable tool for understanding these behavioral effects (Löscher 2002; Grone and Baraban 2015). Pilocarpine, a cholinergic agonist, induces status epilepticus and chronic epilepsy, closely mimicking human temporal lobe epilepsy. This model offers several advantages, including reproducibility, the ability to study the progression of seizures, and the assessment of postictal behavioral changes. Mice subjected to pilocarpine-induced seizures exhibit anxiety-like behaviors, cognitive deficits, and hyperactivity, reflecting the behavioral patterns observed in human epilepsy. These models are instrumental in exploring the underlying mechanisms of epilepsy-induced behavioral changes and in evaluating potential therapeutic interventions. By bridging the gap between preclinical findings and clinical outcomes, pilocarpine models contribute to the development of targeted therapies addressing both seizures and associated behavioral impairments. Results of the study, showed that, CKC through its multi-target activity, exhibit significant pharmacological advantage in alleviating the effects of epilepsy in mice.

Initially, an investigation was carried out to assess the impact of CNC on the behavioral patterns of experimental mice. As shown in Table 1, the administration of CNC caused reduction in the percent of mice that convulsed from 94% (P350 group) to 80% in the 100 mg/kg treated group. It also led to an increase in percentage of survivals 24 h after the 1st convulsion in a dose-dependent manner. Thus, it was suggested that the administration of CNC considerably extended both the latency to the first convulsion and the time to death in comparison to the group that was given pilocarpine with no drug.

Table 1. Ratios and Percentages of Development of Convulsions and Survivals, after Pilocarpine-induced (PILO) Convulsions, in Mice Pretreated with CNC

Observation	Treatment before P350			
	PILO	CNC25	CNC50	CNC100
Number of convulsed animals up to 90 min after P350	33/35 (94%)	25/25 (100)	22/25 (88%)	20/25 (80)
Survivors up to 24 h after P350-induced convulsions	2/33	4/25	6/25	8/25
Latency to 1st convulsion (seconds)	678.2±12.2	734.5±15.3**	965.3±19.2***	1012.5±24.1***
Survival (seconds)	1167±27.5	1422±34.2*	1603±42.5**	1892±51.3***

Note: Latencies to the 1st convulsion and to death are represented by means±S.E.M. (seconds), after P350 administration, and analyzed by Kruskal–Wallis, followed by the Dunn's multiple comparison test. Latency to 1st convulsion: **p<0.01 and ***p<0.001 vs. P350. Latency to death: *p<0.05, **p<0.01 and ***p<0.001 vs. P350. Data on Survivors were analyzed by the nonparametric Log-rank (Mantel–Cox) test (comparison of each survival curve to P350).

The percentage of animals that did not exhibit convulsions was also significantly raised as a result of CNC, which also significantly increased the survival rates of the animals. These findings indicate that CNC exhibited significant anti-convulsive activity. This may be attributed to the bioactive phytosterols and terpenoids present in the extract, which have been reported to exert neuroprotective and membrane-stabilizing effects (Ghosh *et al.* 2014; Kim *et al.* 2015).

The next part of the study was conducted to elucidate the possible mechanism of action of CNC. Thus, the effect of CNC (50 mg/kg and 100 mg/kg) was estimated in combination with atropine (ATRP, a muscarinic antagonist), memantine (MEM, a NMDA-type glutamate receptor blocker), diazepam (DZP, a drug which binds to a specific subunit of the $GABA_A$ receptor).

Some drugs, such as atropine, that block muscarinic acetylcholine receptors may help treat epilepsy by slowing down the brain activity that leads to seizures. This is done by lowering the number of wrong brain messages that could cause seizures, *i.e.* by lowering the activity of these receptors (Aldossary 2022a). For the present study, the mice were given atropine (ATRP) before giving them pilocarpine, a drug that is known to make cholinergic neurons work. In an earlier study, a strong link was found between cholinergic activity and the start of these seizures. This was demonstrated when methylscopolamine, another muscarinic blocker, was given before pilocarpine treatment and effectively weakened most of its effects (Marchi *et al.* 2007a).

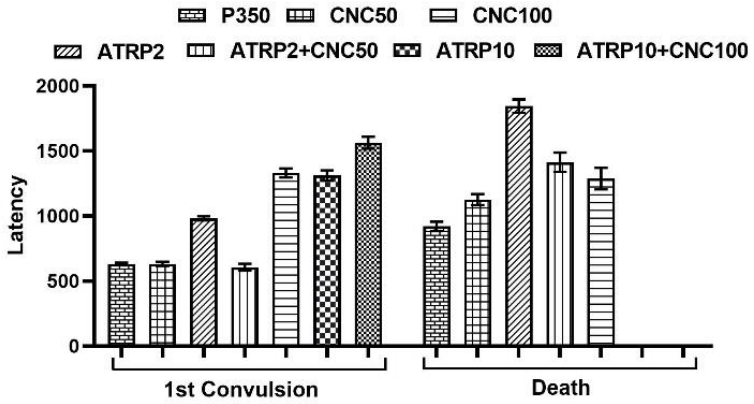

Fig. 1. Effect of CNC in combination with ATRP. The data represent means±S.E.M. for latency to the 1st convulsion and death. No significant differences among treatments were observed (Skillings–Mack non-parametric Two-way ANOVA, followed by the Dwass–Steel–Critchlow–Fligner test for multiple comparisons).

As shown in Fig. 1a, an increase in latency to the first convulsion was observed in P350 group pre-treated with CNC100, or ATRP10 or in the combination of CNC100+ATRP10 in comparison to P350 group. However, no significant difference was observed in the CNC50, ATRP2, or a combination of these. Thus, it was suggested that ATRP helped avoid seizures a little bit, but when it was mixed with another drug (CNC), it didn't make CNC work better at stopping seizures. Atropine can stop seizures caused by pilocarpine by connecting to M1 muscarinic receptors, as shown in earlier research (Hamilton *et al.* 1997). It is thought that these receptors are the main cause of seizures. Previous studies have shown that muscarinic receptor blockade attenuates pilocarpine-induced seizures (Marchi *et al.* 2007b; Aldossary 2022b). However, the present research

showed that muscarinic receptor activation was not the only criterion controlling the anti-seizure effects of CNC. Thus, to further explore the possible mechanism of action of CNC, the pharmacological activity CNC was tested in combination with MEM (McShane *et al.* 2019), to identify the role of glutaminergic receptor in anticonvulsive action of CNC. As shown in Fig. 2, no significant increase in latency to the 1st convulsion was observed in the MEM2+CNC50 combination group, suggesting the memantine was not able to prevent pilocarpine induced convulsion in comparison to P350 treated group. Studies showed that seizures begin and progress with glutamate, and research shows that aberrant glutamate release activates many neuronal populations at once, causing seizures (During and Spencer 1993; Barker-Haliski and Steve White 2015). One possible element in the epileptogenic process and the origin of seizures is changes in glutamatergic transmission *via* the perforant route. Enhanced glutamatergic transmission helped reduce the seizure threshold, according to patch-clamp recordings in rat hippocampus slices taken after pilocarpine-induced status epilepticus (Jakubs *et al.* 2006; Amakhin *et al.* 2018a). In the current study, neither the use of pilocarpine nor the combination of the NMDA receptor antagonist memantine with the CNC increased the effectiveness of the latter. This indicates that the anti-convulsive action of CNC is not mediated *via* NMDA-type glutamatergic receptor antagonism, which is consistent with literature reporting limited efficacy of NMDA antagonists in this model (During and Spencer 1993; Amakhin *et al.* 2018b).

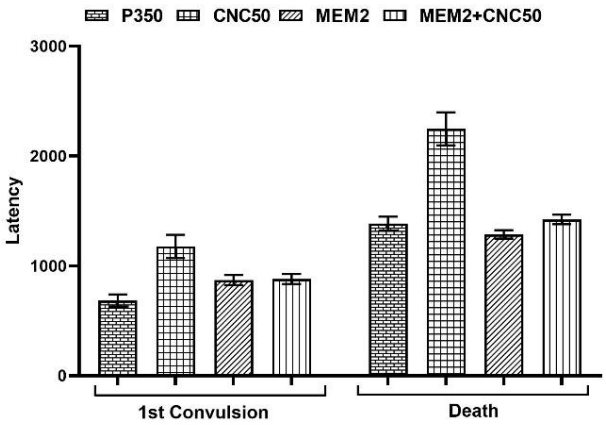

Fig. 2. Effect OF CNC in combination with MEM. The data represent means±S.E.M. for latencies to the 1st convulsion and to death. No significant differences among treatments were observed (Skillings–Mack non-parametric Two-way ANOVA, followed by the Dwass–Steel–Critchlow–Fligner test for multiple comparisons).

The last combination of CNC was performed with DZP to find out the relation of BDZ receptor on the anticonvulsive action of CNC. As shown in Fig. 3, in comparison to P350 untreated group, the P350 pre-treated alone and combination group (CNC50, CNC100, DZP0.2, DZP0.2+CNC50, DZP0.5, and DZP0.5+CNC100) showed significant improvement in the latency to the 1st convulsion. The latency to death, on the other hand, did not show any significant differences between the groups. This is most likely because the majority of the animals did not experience convulsions or die when the CNC and DZP were combined. It has been suggested that the extract may enhance GABAergic transmission, possibly due to phytosterols, which are known to modulate GABA-A receptor activity (Zavvari *et al.* 2018)

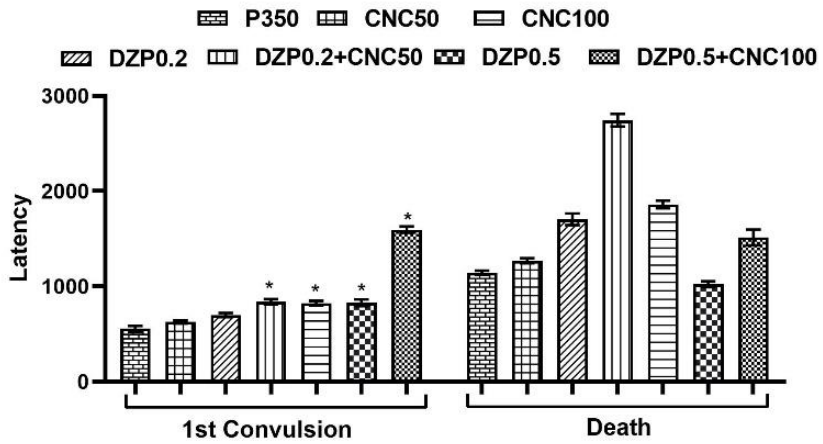

Fig. 3. Effect of CNC in combination with DZP. The data represent means±S.E.M. for latency to 1st convulsion and death. Latency to the 1st convulsion: *p>0.004 to 0.010 vs. P350 (Skillings–Mack non-parametric Two-way ANOVA, followed by the Dwass–Steel–Critchlow–Fligner test for multiple comparisons).

Dopaminergic receptors are the mechanism by which dopamine functions to exert an influence on both inhibitory and excitatory pathways, hence contributing to the susceptibility to and propagation of seizures (Jakubs *et al.* 2006; Kasica *et al.* 2022). In this process, dopamine (DA) and its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), play significant roles in the pathophysiology of epilepsy by modifying neuronal excitability and neurotransmission. It has been established that decreased levels of DA are associated with increased seizure activity, whereas the restoration of DA may have anticonvulsive effects (Chen 2006). DOPAC, which is a measure of DA metabolism, is a reflection of dopaminergic activity and turnover. Changes in its levels have been seen after seizures (Sanz-Novo *et al.* 2023).

It is possible that anticonvulsant medications, such as levetiracetam and valproate, can restore dopaminergic balance and reduce hyperexcitability by modulating the amounts of dopamine and dopamine-related peptides (DA and DOPAC) (Cengiz *et al.* 2024). Studies have shown, for example, that these medications can either boost the release of dopamine (DA) or limit the breakdown of DA, which is correlated with greater seizure management.

Keeping an eye on the levels of DA and DOPAC could provide valuable information regarding the effectiveness of antiepileptic treatments and the dopaminergic mechanisms that are responsible for the management of epilepsy. Thus, next part of the study aimed at determining the level of monoamines, such as Dopamine (DA) and DOPAC (3,4-dihydroxyphenylacetic acid) in the mice striata. As shown in Fig. 4, the level of both DA and DOPAC was found significantly reduced in P350 treated group. However, upon administration of CNC, a dose-dependent increase in both DA and DOPAC was observed. Thus, it was suggested that anticonvulsive action of CNC might be due to modulation of DA generation and prevention of its metabolism which cause increase in striatal DA level. Previous studies have implicated dopaminergic imbalance in epilepsy pathophysiology, with restoration of DA levels shown to reduce seizure susceptibility (Chen 2006; Sanz-Novo *et al.* 2023)

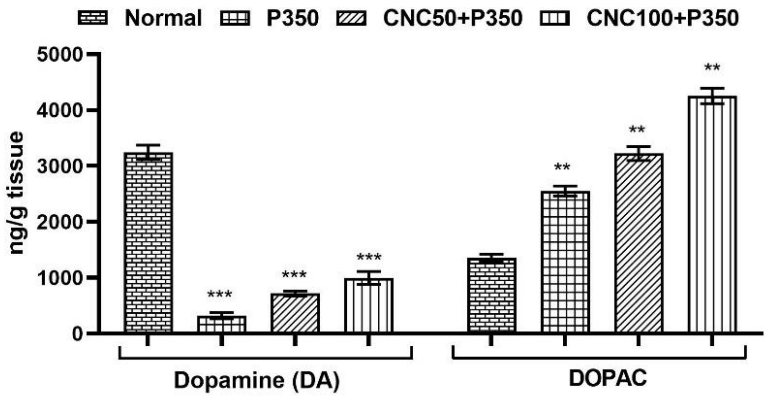

Fig. 4. Effect of CNC on the Dopamine (DA) and DOPAC. The data represent means±S.E.M., analyzed by One-way ANOVA with the Student–Newman–Keuls as a post-hoc test. DA: ***p>0.001 vs. Normal controls; DOPAC: **p>0.01 vs. Normal control.

Oxidative stress is a hallmark of epilepsy and a key contributor to the onset of seizures and subsequent brain damage. This disorder is caused by an imbalance between the brain's antioxidant defence and the generation of reactive oxygen species (ROS) (Aguiar *et al.* 2012). A rise in mitochondrial reactive oxygen species (ROS) and subsequent oxidative damage to DNA, lipids, and proteins occurs during seizures due to the abnormally high levels of neuronal activity that seizures induce. Epilepsy thrives in damaged brain areas because it messes with ion channels, neurotransmitter release, and neuronal signaling. There is a vicious cycle of neuronal hyperexcitability and injury that begins with oxidative stress and continues with mitochondrial dysfunction, which worsens energy deficiencies and keeps ROS generation going (Waldbaum and Patel 2010). The oxidative stress that results from and contributes to the development of epilepsy is brought to light by this process. In this regard, it is worthwhile to assess the effect of CNC on the oxidative stress in the PILO induced mice. Thus, in the next part, the level of various oxidative stress biomarkers was determined, such as MDA, SOD and CAT in the brain cortex and in the blood (Aguiar *et al.* 2012). In epilepsy, elevated thiobarbituric acid reactive substances (TBARS) indicate increased lipid peroxidation due to oxidative stress caused by recurrent seizures. This oxidative damage contributes to neuronal dysfunction and hyperexcitability, worsening seizure pathology (Gluck *et al.* 2000). Monitoring TBARS levels helps evaluate oxidative stress in epilepsy, providing insights into disease progression and the potential antioxidant effects of antiepileptic drugs in reducing neuronal injury. Carbonyl protein levels serve as a marker of oxidative stress in epilepsy, reflecting protein oxidation caused by reactive oxygen species (ROS). Elevated carbonyl proteins are commonly observed in epilepsy, particularly after seizures, due to increased ROS production and impaired antioxidant defences. Oxidative modification of proteins disrupts their structure and function, contributing to neuronal damage, synaptic dysfunction, and seizure progression. Sulfhydryl proteins, nitric oxide (NO), superoxide dismutase (SOD), and catalase (CAT) play critical roles in the pathophysiology of epilepsy by modulating oxidative stress and neuronal integrity (Nobre *et al.* 2009; Shehta *et al.* 2022). Sulfhydryl groups in proteins are essential for maintaining their structure and function. However, seizures generate excessive reactive oxygen species (ROS), leading to the oxidation of sulfhydryl groups, disrupting protein function, and contributing to neuronal damage (Rodrigues *et al.* 2012, 2013). NO, a neuromodulator, becomes neurotoxic during seizures when produced in excess, forming reactive nitrogen species (RNS) like peroxynitrite,

which exacerbates neuronal excitability and injury. SOD, an antioxidant enzyme, neutralizes superoxide radicals, but its impaired or overwhelmed activity in epilepsy allows ROS accumulation, intensifying oxidative stress. Similarly, reduced catalase (CAT) activity during seizures fails to detoxify hydrogen peroxide, further amplifying neuronal damage. Monitoring these biomarkers highlights the oxidative stress burden in epilepsy and offers insights into potential therapeutic strategies, particularly antioxidant-based treatments, to mitigate seizure-induced neurodegeneration (Sudha *et al.* 2001). According to the findings of the current investigation (Fig. 5), the levels of TABARS, carbonyl protein, and nitric oxide were found to have greatly increased, whereas the levels of sulfhydryl protein, SOD, and CAT were found to have decreased in the PILO group. After the administration of CNC, the levels of these biomarkers were dramatically restored to normal levels, with the group that was treated with 100 mg/kg of CNC reporting the highest activity in comparison to PILO untreated-group. This antioxidant effect may be attributed to the presence of terpenoids and phytosterols in the extract, which have been reported to scavenge free radicals and inhibit oxidative damage (Aguiar *et al.* 2012; Sudha *et al.* 2001)

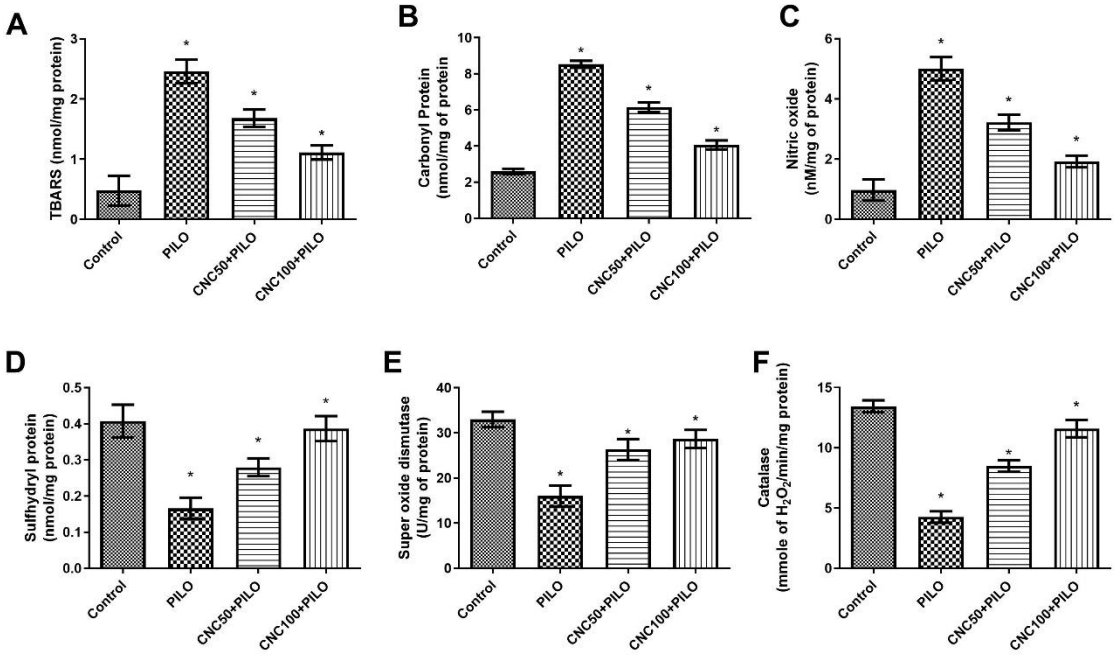

Fig. 5. Effect of CNC on the oxidative stress biomarkers. The data are mean±S.E.M. *P < 0.05 vs control (One-way ANOVA with the Student–Newman–Keuls as a post-hoc test).

CONCLUSIONS

1. The *Callicarpa kwangtungensis* Chun (CNC) extract was found to possesses significant antiepileptic potential in pilocarpine (PILO)-induced epilepsy in mice.
2. One possible explanation for the pharmacological effects of CNC was its role in the GABAergic and DAergic signaling pathways interacting with one another. Additionally, it showed a great deal of antioxidant efficacy in mice.

3. Further investigation into CNC's effects on other receptors, neuronal apoptosis, and the biochemical mediators of apoptosis is necessary to support CNC as a prospective therapeutic candidate.

AUTHOR'S CONTRIBUTION

Shiqing Li, Meizhi Li: design research plan, implement research process, thesis writing; Rutong Wei: put forward research ideas, paper review; Huawen Liu: Implementation of the research process, analysis of experimental data; Erwei Hu and Zhibing Zhou: Implementation of the research process, thesis revision.

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