In vitro anti-inflammatory compounds from Viburnum coriaceum show inhibition of epileptic seizures in rat model

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Abstract

Inflammatory mediators released during seizure act as neuromodulators for nerve cell functioning along with inflammation build-up. They can be targeted for the treatment of epilepsy. Anticonvulsant activity of *Viburnum coriaceum* extract suggested the potential of the plant in neurological disorders. Preliminary analysis revealed the inhibitory potential of plant root-hexane extract on trypsin and LOX *in vitro*. This was then fractionated by various chromatographic techniques for the isolation of active compounds. *In silico* scrutiny of compounds in the GC profile revealed two active compounds, docosane, and methyl palmitate. These compounds were subjected to trypsin and LOX inhibition and their kinetics assays, LOX and COX inhibition assays in LPS induced inflammatory IMR 32 cell lines, and antiepileptic activity on pilocarpine-induced rat models. Docosane showed 50.74% trypsin and 82.51% LOX inhibition. Methyl palmitate showed 91.04% trypsin and 81.12% LOX inhibition. Both the compounds showed a mixed mode of inhibition for trypsin. Docosane showed a mixed-mode and methyl palmitate showed an uncompetitive mode of inhibition for LOX. In the cell culture studies, methyl palmitate had 75.25% and docosane had 67.66% inhibition on COX whereas they had 72.97% and 67.22% inhibition on LOX respectively. Methyl palmitate showed a better antiepileptic activity on rat models.

Introduction

Viburnum coriaceum Blume is a member of the largest clade Viburnums in the Adoxaceae family, which includes around 200 species of small trees and shrubs [1]. Bio-properties of Viburnums are provided by the rich repertoire of compounds present in them. *Viburnum opulus* is a plant with great medicinal importance. *Viburnum coriaceum* has a phylogenetic relationship with *Viburnum opulus* [2] and was least studied in the medicinal aspect. Our initial study revealed the bioactivities possessed by the crude extracts of *Viburnum coriaceum* including anti-inflammatory, antioxidant, antibacterial, etc. [3]. Formulations of *Viburnum coriaceum* were reported to have anticonvulsive activity suggesting the neurological effects of the plant [4]. neurodegenerative disorders, epilepsy, autism, obesity, etc. are the worst consequences of inflammation. The blood-brain barrier (BBB) is believed to be protecting the central nervous system (CNS) from the immune cells and other factors, thus helping to maintain its homeostasis [5]. BBB alterations occur in various systemic infections, and inflammations affect its permeability and in turn the homeostasis of the CNS [6]. The neurotoxic factors released during inflammation aid the persistence of inflammatory stimuli which cause degeneration in the CNS [7]. The probability of these consequences would increase with age. The designation "neurogenic neuroinflammation" is given for the inflammatory reactions, followed by neuronal activities like excitability and seizures [8]. Several inflammatory mediators are released by the nerve cells under excitability and seizures. They function as neuromodulators which have a direct effect on the nerve cell functions along with their role in inflammation build-up [9]. Hence neuroinflammatory mediators can be targeted for the treatment of epilepsy and other neurological disorders [10].

The present study focused on isolating secondary metabolites from the plant extracts, having the potential to inhibit enzymes that play major roles in inflammatory pathways. The serine protease, trypsin

is selected as the main target which acts through proteinase-activated receptors (PAR). PARs sense the proteinases and control their release by feedback mechanism [11]. PAR-2, a subtype of PAR, is commonly activated by trypsin, has major involvement in many inflammatory diseases like cardiovascular, gastrointestinal, neurological, and pulmonary diseases [12]. The level of PAR 2 and its agonist trypsin are found to increase in many inflammatory disorders. In brain tissues, an increased expression of this receptor and its activator is seen during neurodegenerative disorders and epileptogenic seizures [13]. Hence inflammatory pathways regulated by proteinases and PAR can be targeted for the consequences of neuroinflammation [14]. The study also included lipoxygenase (LOX) and cyclooxygenase (COX), two major enzymes in arachidonic acid metabolism, since the inflammatory operators/mediators like leukotrienes, prostaglandins, and thromboxane biosynthesis are mediated by LOX and COX [15]. Finally, the present study was focused on the inhibition of peripheral inflammatory parameters and their consequences on the onset of epilepsy. Compounds from the hexane extract of *V. coriaceum* were studied for their impact on elevated peripheral inflammatory mediators and convulsions developed by pilocarpine and severity in status epilepticus. It was hypothesized here that the compounds may have a role in the reported antiepileptic property of the *V. coriaceum* extract [4]. Protease-activated receptor-2 mediates in reducing seizures by regulating trypsin expression [16]. The lipoxygenase inhibitors reported with anticonvulsant activity supported our hypothesis [17].

Methods

Plant material collection and preparation

The authors procured permission from the Forest Department, Govt. of Kerala, India to visit and collect the plant sample from the forest areas for research purposes as per Order No. WL 10-47474/2014 dated 1/12/2015 and accordingly procured the sample for the study. Plant materials were collected from the Nilgiri Hills, India during the month of August, were dried under shade and powdered. Root and aerial parts were powdered separately. The material was identified and authenticated by Dr. Prabhukumar K. M., Scientist and Head, Plant Systematics and Genetics Division, Centre for Medicinal Plants Research, Kottakkal, India. Root and the aerial parts were extracted with hexane, methanol and water successively. All the fractions were concentrated and stored for further studies. All study related to plant were performed in accordance with the relevant guidelines and regulations.

Selection of the extracts

Out of the different extracts studied, hexane extract of the plant root showed higher inhibitory activity on the enzymes selected for the study [3].

In vitro **anti-inflammatory assays**

Trypsin inhibition assay

A continuous rate spectrophotometric method, developed by Sigma Aldrich with slight modifications was used for the assay [3]. The substrate used was 0.25 mM N_{α}- benzoyl- L- arginine ethyl ester (BAEE) in

phosphate buffer at pH 7.6. One BAEE binding unit of trypsin will show an absorbance of 0.001 per minute at pH 7.6. The enzyme (0.05 mM) was prepared in ice-cold 0.001 M hydrochloric acid and the inhibitor in dimethyl sulfoxide (DMSO). Inhibitory action was expressed as the number of BAEE units of trypsin inhibited by the inhibitor and it was expressed as a percentage of inhibition. The reaction mixture contained a total volume of 3.4 mL with a 3 mL substrate, 0.2 mL enzyme, and 0.2 mL inhibitor. Enzyme and the inhibitor were incubated for 10 minutes. Then, the substrate was added and the readings were taken at 253 nm by time scanning for 10 minutes. 0.1 mM concentration of phenylmethyl sulfonyl fluoride (PMSF) was used as the standard inhibitor.

Blank → 0.2 mL DMSO, 0.2 mL HCl and 3 mL substrate

The activity of the native enzyme (N) → 0.2 mL DMSO, 0.2 mL enzyme, and 3 mL substrate

Activity of the inhibitor (I) → 0.2 mL inhibitor, 0.2 mL enzyme, and 3 mL substrate

Percentage of inhibition = $\left(\frac{N-I}{N}\right)x100$

LOX inhibition assay

LOX inhibition assay, developed by Anthon and Barrett with slight modifications was used [3]. LOX (EC 1.13.11.12) type I-B (soybean) was the enzyme used and linoleic acid was the substrate. 0.93 µM solution of enzyme and 0.32 mM substrate was prepared in 0.2 mM borate buffer at pH 9. The assay mixture contained 50 µL enzyme, 360 µL substrate, and 1.59 mL borate buffer to make the final volume of 2 mL. While adding inhibitor (50 µL) to the reaction mixture, buffer volume had to be reduced to 1.54 mL to maintain the total volume at 2 mL. The formation of hydroperoxyl-octadecadienoic acid was the indicator of LOX activity, which could be measured in a spectrophotometer at 234 nm. Vanillin of 1 mM was used as the standard inhibitor.

Blank → 50 µL DMSO, 1.59 mL buffer, and 360 µL substrate

Activity of the native enzyme (N) → 50 µL enzyme, 50 µL DMSO, 1.54 mL buffer, and 360 µL substrate

The activity of the enzyme in the presence of inhibitor (I) → 50 µL enzyme, 50 µL inhibitor, 1.54 µL buffer, and 360 µL substrate

Percentage of inhibition = $\left(\frac{N-I}{N}\right)x100$

Activity guided isolation of the compounds

Hexane extract of the plant which showed a very high inhibition of trypsin and LOX were subjected to fractionation by column chromatography using silica gel of 100–200 mesh size. The sample was loaded and eluted with a hexane-acetone solvent system by gradient elution. 25 fractions of 25 mL each were

collected and subjected to enzyme inhibition assay. Active fractions were pooled and further fractionated with a column of 230–400 mesh size. The same solvent system was used for elution. 25 fractions were collected and analyzed for enzyme inhibition. Active fractions were pooled and spotted on analytical thin-layer chromatographic plates to know the banding pattern. Similar ones were pooled and purified by preparative thin-layer chromatography.

GC-MS analysis of the purified band

Characterization of the active fraction was done by GC-MS. Analysis was performed using GCMS-QP2010 Shimadzu Gas Chromatograph and Mass Spectrometer. The phytochemical profile of the fraction was created by GC 2010 Gas chromatography. 99.9% helium gas was the carrier gas with a flow rate of 1 mL per minute and the sample was injected by splitless mode with an injection volume of 1 µL and injection temperature of 260°C. The column temperature was initially programmed at 80°C for 4 minutes and then allowed to increase linearly to 280°C with a hold time of 6 minutes. The GC-MS ion source temperature was 200°C and the interface temperature was 280°C. The compounds in the sample were identified by comparing the retention time, mass spectra, and fragmentation pattern. NIST library data were used for the reference.

In silico **analysis of the enzyme-compound interaction: Preparation of the protein and ligands**

Interaction of the compounds, identified by the GC-MS analysis, with the enzyme was studied by *in silico* molecular modelling and docking techniques. This was performed using Schrodinger Maestro 10.4 software package. The protein crystal structure coordinates for docking studies were downloaded from the PDB with the ID of trypsin, 4MTB. The initial step in the protein preparation protocol was to remove the water molecules from the crystal structure and addition of polar hydrogen groups. Then the structure was minimized at an RMSD (root mean square deviation) cut-off of 0.30 Å using protein preparation wizards. The force field assigned was OPLS 3e [18]. A grid of 20 Å dimensions was set with the centre of the crystallographic ligand as the centre of the grid. Ligand molecule's structures were downloaded from the PubChem Database and were prepared for docking studies using LigPrep module of the Schrodinger program, which produces the lowest energy conformations. All the possible conformations of the compounds were generated at pH 7. Docking studies were performed by the extra precision method and the binding energies were predicted with prime MM/GBSA.

Selection of compounds

The compounds with the highest glide score for trypsin inhibition were selected and purchased from Sigma Aldrich for further studies. As an initial step, the enzyme (trypsin and LOX) inhibitory potential of pure compounds (1 mM) was carried out by the same procedure described above.

Enzyme kinetics assay for trypsin and LOX

The enzyme-ligand reaction kinetics was found out by enzyme kinetics assay [19]. The same protocol for enzyme inhibitory assay was followed here with different substrate concentrations. The rates of the reaction at different substrate concentrations and the mode of inhibition were also identified by this

assay. The different substrate concentrations used for trypsin were 0.03125, 0.0625, 0.125, 0.25, 0.5 mM, and for LOX 20, 40, 60, 80 and 100 µM. The activity of the enzyme at different substrate concentrations was measured first and the same was repeated with 1 mM concentration of the compounds of study. Change in the specific enzyme activity against the respective substrate concentrations was plotted and a Lineweaver-Burk (LB) plot was drawn. From the plot Michaelis-Menten constant (K_m) and the maximal velocity (V_{max}) were determined. The inhibitor constant (K_i) was calculated with

$$K'_m = K_m \left(1 + \frac{I_O}{K_i}\right)$$

K′$_m$ is the Michaelis-Menten constant for the enzyme-inhibitor complex.

The Cheng-Prusoff equation was used to calculate the IC_{50}

$$K_i = \frac{IC_{50}}{(1 + \frac{[S]}{K_m})}$$

Anti-neuroinflammation assay

Cell culturing and inflammation induction

IMR 32 cells were procured from National Centre for Cell Sciences (NCCS), Pune, India. IMR 32 cells are continuous hyper-diploid cell lines from human neuroblastoma tissue [20]. These cell lines are used for the study of various neurological disorders like neuro-inflammatory diseases, Alzheimer's disease, etc. The cells were then maintained in Dulbecco's modified eagles' medium (DMEM) of Sigma Aldrich. Subculturing of the cells was done in a 25 cM^2 tissue culture flask containing DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany), and antibiotic solutions, Penicillin (100 U/mL), Streptomycin (100 µg/mL), and Amphotericin B (2.5 µg/mL). Cultured cell lines were kept at 37 ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany). The cells grown with 60% confluency were treated with 1 µL of 1 µg/mL lipopolysaccharide (LPS) for inducing inflammatory reactions. LPS activated cells were then treated with different concentrations of the inhibitor (25, 50, 100 µg/mL) and incubated for 24 hours. After incubation, the anti-inflammatory assays were performed using the cell lysate.

COX activity

The COX inhibitory activity was determined by the method of Walker and Gierse [21]. 100 µL cell lysate was mixed with Tris-HCl buffer of pH 8, glutathione 5 mM/L, and haemoglobin 5 mM/L. The mixture was then incubated for 1 minute at 25°C. 200 mM/L of arachidonic acid was added to initiate the reaction and 200 µL 10% trichloroacetic acid in 1 N hydrochloric acid was added to terminate the reaction after 20

minutes of incubation at 37°C. The mixture was centrifuged, 200 µL of 1% thiobarbiturate was added, boiled for 20 minutes, and allowed to cool. This was again centrifuged for three minutes. COX activity was determined by reading absorbance at 632 nm.

Percentage of enzyme inhibition = $\left(\frac{N-I}{N}\right)x100$

N - Activity of the native enzyme

I - Activity of the enzyme in the presence of inhibitor

LOX activity

LOX activity was determined by a method developed by Axelrod [22]. 2 mL reaction mixture contained 50 µL of the cell lysate, 200 µL sodium linoleate, and Tris HCl buffer at pH 7.4. The LOX activity was monitored by measuring absorbance at 234 nm, which reflected the presence of 5-hydroxyeicosatetraenoic acid.

Percentage of enzyme inhibition = $\left(\frac{N-I}{N}\right)x100$

N - Activity of the native enzyme

I - Activity of the enzyme in the presence of inhibitor

Antiepileptic study on rat models

Ethics approval statement

Al the experiments were carried out according to the guidelines of the Committee for Control and Supervision of Experiments on Animals (CPCSEA), India and ARRIVE (Animal Research: Reporting of In Vivo Experiments) after getting approval from the Internal Animal Ethical Committee of Kannur University, Kerala, India (KULS/IAEC/2017/19).

Toxicity study

The study was performed according to the guidelines of the Organisation for Economic Co-operation and Development (OECD). The doses selected for the study were 500 mg/kg and 1000 mg/kg. Animals were divided into five groups with six members each. One group was kept as control and two groups for two doses of each compound. Animals were kept in a fasting condition overnight with access only to water before the experiment. After the administration of compounds animals were kept under 48 hours of observation for any toxic effects [23].

PILO induced convulsions - Animals

Sprague Dawley rats (200–250 g) of either sex were obtained from Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India. The randomly selected animals were maintained in the Animal House facility of the Department of Biotechnology and Microbiology, Kannur University in polypropylene cages at room temperature with free water and food access. After one week of acclimatization, experiments were carried out according to the guidelines of the Committee for Control and Supervision of Experiments on Animals (CPCSEA), India (KULS/IAEC/2017/19).

Drugs and Chemicals

Analytical grade methyl scopolamine (MS), pilocarpine (PILO), diazepam (DZ), docosane (DC), and methyl palmitate (MP) were bought from Sigma Aldrich.

Induction of Status Epilepticus

350 mg/kg of PILO was injected i.p. on the drug-treated animals to induce convulsions. Pretreatment with testing drugs (DC and MP) and standard drug (DZ-10 mg/kg) was done before 30 minutes of PILO injection. Dosages chosen for DC and MP were 100 mg/kg and 200 mg/kg. This was based on a minimum dose with maximum activity. Peripheral effects of the inducer were reduced by i.p. injection of MS (1–2 mg/kg). Animals were kept in observation cages for 2 hours to record latency, onset, and different stages of Status Epilepticus (SE) [24]. 7 groups of 6 animals each were chosen. The first group was kept as blank (normal rats), second-negative control (MS and PILO), third-positive control (MS, DZ, and PILO), and the other four groups were test groups. Groups 4 and 5 were DC test groups and groups 6 and 7 were MP test groups.

Behavioral stages of SE

Animals were observed for 2 hours closely after the injection of PILO for rating behavioral changes, measuring latent period and the onset of SE. The rating was done based on the Racine score [25]. Different stages of seizure according to the Racine score are stage 0 (baseline)-without any behavioral changes, stage 1-absence seizure-like immobility, stage 2-head nodding, facial movements with or without salivation, stage 3-clonuses in forelimbs, slight rearing with excess salivation, stage 4-rearing and falling and stage 5-generalized convulsions (tonic-clonic seizures). The first three stages were considered as non-convulsive seizure (NCS) stages and the other three as convulsive seizure (CS) stages [26].

Blood sampling and analyses

Animals were anesthetized with isoflurane and blood samples were collected from the right heart ventricle by cardiac puncturing during SE and after 24 hours of SE. The samples were collected in ethylene diamine tetra acetic acid (EDTA) tubes for cell count analysis and clot activating tubes for separating plasma. Plasma was separated by centrifugation at 3000 rpm for 10 minutes at 4°C and was analyzed for inflammatory markers during epileptogenesis [27]. Considering the role of PILO in triggering inflammatory parameters and in turn seizure activities, three important factors from blood were considered for the study: WBC count with the percentage of lymphocytes, IL-1β, and CRP levels.

WBC total and differential count

Total and differential counts of WBC were estimated using the impedance method (Horiba Pentra ES 60). Total count was measured in number per cubic millimeter and differential counts were expressed in percentage.

IL-1β quantification

Plasma concentration of IL-1β was quantified by enzyme-linked immune sorbent assay (ELISA) (KB31165 IL-1β rat analyte kit, Krishgen biosystems, India) as instructed in the manual.

CRP quantification

Plasma concentration of C-reactive protein (CRP) was quantified by ELISA (88-7501 Rat CRP kit, Thermo Fisher Scientific) as per the instructions in the manual.

Brain tissue isolation, preparation, and analyses

Animals were killed by decapitation for isolating brain tissues during SE and after 24 hours of SE. The brain was removed and dissected within 4 minutes after decapitation at -18°C on a cold plate. The Hippocampus, the central region for the development of seizures in the PILO model [28], and the choroid plexus was isolated from the brain to measure the serum albumin content as an indicator of BBB permeability.

Dissection was started by removing the fur and attached skin above the cranium. The cranium was opened carefully from the foramen magnum and after removing calotte, the brain was taken out *in toto*. Before isolation, a cold plate was set at a temperature of -18°C. All the dissection tools were also maintained at the same temperature. The first part to be separated was olfactory bulbs. Further steps were followed as per the dissection lines by Zeman and Innes. A transverse incision was made at A_{22} to separate the brain into dorsally located and caudally located parts. Then incision was made at a position below A_{17} to get a tissue slice where the hippocampus was visible and can be easily separated [29]. The isolated hippocampus and the rest of the tissues were stored at -80°C till it was analyzed [30].

Before choroid plexus isolation, blood vessels from the dorsal and ventral regions of the brain were removed. The choroid plexus was isolated by placing the brain in the dorsal side up position and fixing it in place using large forceps. A smaller forceps was inserted through the midline at the central part till the top of the midline of the hippocampus, punctured through the cortex and corpus callosum, and pulled away to expose the region. It was identified by the wavy red line and separated by pulling the end using a small forceps or needle. The same procedure was repeated to remove the choroid plexus of the other hemisphere [31].

Determination of the serum albumin content in the choroid plexus

The choroid plexus isolated during SE was weighed and mixed with barbital buffer at pH 8.6 for homogenization. The homogenate was centrifuged for 20 minutes at 2000 rpm. The supernatant was

used for determining the albumin content. The assay was carried out using an albumin ELISA kit (E111-125, Bethyl laboratories) [32].

Quantification of Glutamate and GABA in the hippocampus

Hippocampus tissue isolated during SE was washed with 0.9% saline to remove the attached blood clots and other tissues. After adding 0.1 N HCl in 80% ethanol to the tissues at a concentration of 1 mL solvent for 100 mg tissue, it was homogenized thoroughly and centrifuged at 4500 rpm for 20 minutes at 25°C. The supernatant was collected and stored for further analysis. Standards of glutamate and GABA were also prepared in 0.1 N 80% HCl. Working standards of 5 to 50 µg/mL were prepared from a stock solution of 1 mg/mL. 2 µL of the samples and standards were applied on pre-coated thin layer chromatography (TLC) plates (Silica gel $F_{254,}$ Merck) and dried at 60–65°C in a hot air oven. Plates were then developed using the mobile phase n-butanol: glacial acetic acid: water (22:3:5 v/v/v) and dried again at 60–65°C. 0.2% ninhydrin in acetone was sprayed and kept at 60–70°C for 5 minutes. The observation was done at 550 nm [33].

Statistical analyses

Statistical analyses were performed using SPSS software (Version 20) and the final data was presented as mean ± SEM. All data were evaluated by one-way analysis of variance (ANOVA) and post hoc comparison test and a value of $P < 0.05$ was taken as significant.

Results

In vitro anti-inflammatory assays and activity guided isolation of the compounds

The enzyme inhibitory activity of the plant extracts showed variation with the solvents of extraction and the plant parts used. Plant roots showed a comparatively high inhibition than aerial parts. Among the different extracts, the root-hexane extract showed the highest account of inhibition for trypsin. In the case of LOX, the maximum inhibition was shown by methanol extract, followed by hexane extract [3]. The inhibition was found to increase linearly with the increase in the concentration of the extracts for both the enzymes (Fig. S1a & b). Root-hexane extract with 95.26% trypsin inhibition and 72.61% LOX [3] inhibition was further fractionated by column chromatography. Fractions 16 to 18 from the silica column of 230–400 mesh size with high trypsin inhibition were pooled. Analytical thin-layer chromatographic plates (Fig. S2) showed a fluorescent light blue coloured band with an Rf value of 0.51 in the fractions when observed under UV (360 nm). The band was purified and isolated by preparative thin-layer chromatography (Fig. S2c).

The GC-MS analysis of the purified band showed the presence of 23 different compounds in it Fig. S3). The compounds were identified by comparing their MS/MS spectra with the compounds in the NIST library. The identified compounds were further scrutinized by *in silico* molecular docking with target enzymes to get down to promising compound/s. The MS/MS spectra of the two compounds selected for further studies are shown below (Fig. S4).

In silico analysis of the compounds

All the compounds from the GC profile were docked with enzyme trypsin. Docking studies (Figs. 1 & 2) revealed the mode of reaction and the binding pattern of the compounds with the active site of the specific enzyme. Compounds were first listed according to their glide scores and the first five compounds with the topmost positions with the glide scores were chosen. They were not differing significantly with respect to their glide scores. Hence, considering their availability in the market two were selected and purchased from Sigma-Aldrich for further studies. Docosane (DC) (Pub Chem ID: 12405), the first selected compound had a retention time of 38.59 and methyl palmitate (MP) (the second selected compound, Pub Chem ID: 8181), had a retention time of 28.427. Both the compounds showed a better interaction with trypsin in the extra precision method of molecular docking. The glide scores of DC and MP were − 3.56365 kcal/mol and − 2.9041 kcal/mol respectively and binding energies were − 25.0553 kcal/mol and − 22.6664 kcal/mol respectively.

Trypsin inhibition kinetics

The trypsin inhibition of MP was shown to have a higher value compared to DC at 1 mM concentration (Fig. 3). Mode of inhibition and the interaction of ligands with enzyme were analyzed by enzyme kinetics studies. LB plot was drawn by taking the values of $1/V_{max}$ and I/[S] in the Y and X-axis respectively. The LB plot of DC (Fig. 4) showed that it had a mixed-mode of inhibition and the V_{max} of the enzyme was found to decrease when it was bound by the inhibitor. The V_{max} values of the enzyme with inhibitor and without inhibitor were 0.083 and 0.047 mM/mg/min respectively. Also, K_m and K'_m values were 0.09 mM and 0.142 mM respectively. Using V_{max} and K_m values inhibitor constant K_i and IC_{50} were calculated and the values were 1.73 mM and 11.33 mM respectively. MP (Fig. 5) also showed a mixed-mode of inhibition and the V_{max} was found to decrease from 0.117 to 0.057 mM/mg/min when the enzyme was bound by the inhibitor. K_m and K'_m values were 0.25 mM and 0.4 mM respectively. K_i and IC_{50} were calculated to be 1.66 mM and 8.3 mM respectively.

LOX Inhibition kinetics

Both the compounds were found to possess very high inhibitory action on the enzyme when compared to the standard inhibitor (Fig. 6). Kinetics studies of the compounds with LOX gave two different modes of interaction. LB plot of DC (Fig. 7) showed a mixed-mode of inhibition. The V_{max} values were 2 mM/mg/min and 0.1538 mM/mg/min respectively for the enzyme with inhibitor and without inhibitor. K_m and K'_m values were 12.34 mM and 181.82 mM, K_i and IC_{50} were calculated to be 0.0728 mM and 0.0733 mM respectively. In the case of MP (Fig. 8) the mode of inhibition was uncompetitive and the V_{max} values were 2 and 0.045 mM/mg/min, showing a decreasing pattern. K_m and K'_m values were 12.34 mM and 500 mM respectively. The inhibitor constant K_i was calculated to be 0.253 mM.

Anti-neuroinflammatory assay: COX and LOX activity

Two compounds showed a remarkable inhibition in the activity of the enzymes studied. At the highest studied concentration (100 µg/mL), MP had 75.25% inhibition and DC had 67.66% inhibition on COX whereas they had 72.97% and 67.22% inhibition on LOX respectively (Fig. 9).

Antiepileptic study on rat models

Toxicity studies

No mortality or symptoms of toxic effects were observed during the experiment period in terms of locomotion, behavior, breathing, body temperature, and food and water intake for the compounds at two doses (500 mg/kg and 1000 mg/kg). Hence, the LD_{50} would be > 1000 mg/kg for both compounds.

PILO induced convulsions

All the test and control groups after treating with drugs and other chemicals were kept ready for further analyses.

Behavioral stages of SE

Within 10–15 minutes of PILO injection, all animals in the negative control group became motionless with piloerection. This was followed by orofacial movements with salivation, red torn eyes, and twitching. This lasted for 25–30 minutes. Towards the end of the stage, forelimb clonus had started with more salivation which was later turned into rearing and falling. 50% of animals showed severe tonic-clonic seizures and died within 3 hours. Animals in this group showed all the stages of the Racine score (Fig. 10; Table 1).

In the animals treated with the standard drug and second dose of MP, behavioral stages reached only stage 2. No types of clonuses were observed in them and they returned to the normal condition within 4–5 hours of injection. In all other test groups, rats reached the 5th behavioral stage but mortality was observed only in the groups treated with the first dose of DC (Table 1).

The latent period and the onset of SE also showed that the effects of the second dose of MP were significant to that of the standard drug. No SE was observed in both cases. In the negative control group, the latent period was 27 ± 7 minutes, groups treated with the first and second dose of DC had 30 ± 15 minutes and 45 ± 7 minutes of latent period respectively and in groups treated with the first dose of MP, the latent period was found to be more than 1 hour (60 ± 6 minutes) (Table 1).

Table 1
Observational studies after pilocarpine injection during SE

Parameters	Group 1 (Normal)	Group 2 negative control (MS, PILO)	Group 3 positive control (MS, DZ, PILO)	Group 4 DC- 1st dose	Group 5 DC- 2nd dose	Group 6 MP- 1st dose	Group 7 MP- 2nd dose
Latent period (min)	Nil	27 ± 7	No SE	30 ± 15	45 ± 7	Above 1 h 60 ± 6	No SE
Racine score	Nil	Stage 5 50% mortality	Stage 2	Stage 5 33.3% mortality	Stage 5	Stage 5	Stage 2

Blood sampling and analyses

WBC count with the number of lymphocytes, IL-1β, and CRP levels was analyzed (Table 2). WBC count was significantly low within 1 hour of PILO injection. Measurement of lymphocyte percentage indicated that the lower count of WBC was by the reduction in the number of lymphocytes. An inverse relation existed between WBC count and IL-1β levels. When the WBC count was lowered with PILO injection, IL-1β levels were elevated from the baseline level. Treated animals showed a positive response with the second dose of MP in the lead. CRP levels did not show any significant variations. Also, there were no significant differences observed in positive and negative control groups when the blood parameters were compared.

Table 2: Changes in the blood parameters during SE and 24 hours after SE

Groups Factors	1 (Normal)	2 Negative control (MS + PILO)	3 Positive Control (MS + PILO + DZ) DZ-10 mg/kg	4 DC-1st dose 100 mg/kg	5 DC-2nd dose 200 mg/kg	6 MP-1st dose 100 mg/kg	7 MP-2nd dose 200 mg/kg
1 hour after PILO injection							
WBC/ mM^3	8048 ± 1000	2593 ± 652	3664 ± 505	3540 ± 498	5796 ± 747	4359 ± 616	7088 ± 452*
Lymphocyte (%)	81.5 ± 5.4	19.2 ± 2.8	22.5 ± 3.0	25.01 ± 3.4	49.6 ± 7.8	31.6 ± 5.0	72.8 ± 4.9*
IL − 1β (pg/mL)	49.9 ± 3.2	203.0 ± 6.9	182.3 ± 11.0	158.3 ± 6.6	123.5 ± 4.8	145.0 ± 5.5	72.4 ± 3.5
CRP (pg/mL)	< 2000	< 2000	< 2000	< 2000	< 2000	< 2000	< 2000
24 hour after PILO injection							
WBC/ mM^3	> 7300	> 7300	> 7300	> 7300	> 7300	> 7300	> 7300
Lymphocyte (%)	> 72	> 72	> 72	> 72	> 72	> 72	> 72
IL − 1β (pg/mL)	< 62	< 62	< 62	< 62	< 62	< 62	< 62
CRP (pg/mL)	< 2000	< 2000	< 2000	< 2000	< 2000	< 2000	< 2000
*No significant difference with the normal rats ($p < 0.05$)							

Determination of the albumin content

Choroid plexus of rat brain from the negative and positive control groups, 1st and 2nd doses DC treated groups and 1st dose MP treated group was found to possess a small concentration of serum albumin. No albumin was detected in the normal rats and rats treated with 2nd dose of MP (Table 3).

Table 3: Quantification of serum albumin glutamate and GABA in brain tissue homogenate

Factors	Group 1 (Normal)	Group 2 (Negative control)	Group 3 (Positive Control) DZ-10 mg/kg	Group 4 DC – 1st dose 100 mg/kg	Group 5 DC – 2nd dose 200 mg/kg	Group 6 MP – 1st dose 100 mg/kg	Group 7 MP – 2nd dose 200 mg/kg
Albumin (ng/mL)	nil	> 1000	933.3 ± 6.8	963.3 ± 9.01	930.84 ± 8.11	921.32 ± 7.8	nil*
GABA (ng/mg)	≤ 270	≤ 270	≤ 270	≤ 270	≤ 270	≤ 270	≤ 270
Glutamate (ng/mg)	326 ± 23.9	504 ± 16.5	330 ± 16.6*	470 ± 12.3	430 ± 24.3	440 ± 20.1	362 ± 24.9*
*No significant difference with the normal rats ($p < 0.05$)							

Quantification of Glutamate and GABA in the hippocampus

There was no significant difference observed in the concentration of inhibitory neurotransmitter GABA in the rat brain after PILO injection and under normal conditions whereas excitatory neurotransmitter glutamate was found to increase from the baseline level. Treated groups showed significant variations in the glutamate level compared to the control (Table 3).

Discussion

The focus of the study was on the hexane extract of *V. coriaceum* root, which showed a high inhibition on trypsin and LOX, for isolating active principles. Various chromatographic techniques were employed for the purification of active fractions from the extract. The presence of 23 different compounds in the active fraction was revealed by GC-MS analysis. *In silico* analysis of the compounds with trypsin was the strategy employed for the selection of compounds. DC is a straight-chain plant metabolite with 22 carbons. Its molecular weight is 310.6 g/mol. It is a colorless, odorless compound with a 369°C boiling point and 44.4°C melting point. It has been reported to be used in food additives, flavoring agents, and fragrances (PDB ligand ID-12405). MP is the other compound selected for the study, which is a methyl ester of palmitic acid. Its molecular weight is 270.5 g/mol, the boiling point is 417°C and the melting point is 30°C. The compound was reported to be used in food additives, cosmetics, cleaning agents flavoring agents, fragrances, etc. (PDB ligand ID 8181). Physiological roles of MP in vasodilation, neuroprotection, immunomodulation, and the anti-apoptotic effect were also reported [34–37].

DC was found to be an important component of *Tamarix boveana* volatile oil which exhibits a high antibacterial activity [38]. Essential oils of *Metaplexis japonica* with antibacterial and antioxidant activity also possess DC as an important component [39]. Anti-inflammatory activity of the *Gaultheria*

procumbens extracts with DC was also reported especially on LOX [40]. Likewise, MP was also an important component of some plant extracts with different bioactivities [41, 42]. Anti-muscarinic activity and anti-inflammatory activity of MP had also been reported elsewhere [43, 44]. All this information helped and supported the current study on anti-inflammatory activities. The compounds' inhibition potential on trypsin (Figs. 1, 2, 3, 4 & 5) and LOX (Figs. 6, 7 & 8) *in vitro*, and on LOX (Fig. 9a) and COX (Fig. 9b) in IMR 32 cells of neuro-inflammation were analyzed to prove their anti-neuroinflammatory potential too.

Neuro-protective effects of anti-inflammatory compounds were established in animal models of Parkinson's disease and other neurological disorders. Most of the anti-inflammatory studies were mainly focused on the prostaglandin pathway and its important enzymes such as COX and LOX [45]. COX and LOX can elicit toxicity to neurons in neurological disorders and are subjects of study for decades. There exists an extensive relationship between the immune system and the nervous system. Targeting inflammatory pathways modulated by proteases also might open new treatment regimes in neurological disorders. Elevated levels of PAR and proteases in many neurological disorders are aiding that thought [46]. PARs and their activators are widely identified for their importance in the development of chronic inflammatory diseases, either peripheral or nervous. The current study identified two plant metabolites with inhibitory potential on trypsin and LOX *in vitro* and LOX and COX in neuronal cells with LPS induced inflammation.

The antiepileptic activity of the compounds with anti-inflammatory activity was mainly due to their effects on peripheral inflammatory factors and in turn BBB permeability and seizure development. Elevated IL-1β level is a key feature of all inflammatory reactions. Elevated serum IL-1β level is an etiological agent responsible for the damage of BBB [47] and the development of pro-convulsive activities. In the current study, the effects of PILO in the blood parameters associated with inflammatory pathways were analyzed initially to understand how the inflammatory markers were regulated by the compounds under study. Serum albumin in the brain was assayed as a marker for detecting the permeability of BBB [48]. Effects of PILO in the excitatory and inhibitory neurotransmitters in the brain during SE were analyzed to detect the effects of compounds under study in regulating the neurotransmitter levels at the SE. Behavioral stages in the development of SE were also considered important for measuring the severity of the condition.

The PILO model is highly isomorphic to human conditions and is a common laboratory model. PILO being a cholinergic muscarinic receptor agonist can activate the inflammatory pathways, thereby increasing the level of proinflammatory mediators. The important characteristics of the PILO model are rapidly induced SE, the appearance of the latent period with spontaneously recurrent seizures, widespread or localized lesions at different regions of the brain, re-organization of neuronal networks in the affected regions of the brain and failure to be completely controlled by antiepileptic drugs [49]. The peripheral pro-inflammatory effects of PILO are also found to be a mechanism behind the development of SE. A fourfold increase in the serum pro-inflammatory cytokine interleukin-1β (IL-1β), a significant decrease in the number of CD-4 lymphocytes, and blood-brain barrier (BBB) damage were observed shortly after the

administration of PILO [50]. Elevated serum IL-1β level is an etiological agent responsible for the damage of BBB and the development of pro-convulsive activities [47]. This has been proved by using an IL-1 receptor antagonist (IL-1Ra), which inhibits the action of IL-1β in biological systems. Studies on the anticonvulsive action of IL-1Ra support the data also [51]. Infections and infestations that affect CNS or peripheral systems evoke inflammatory reactions and in turn, result in seizures and epilepsy [52]. Hence, compounds with anti-inflammatory potential could reduce convulsions by inhibiting pro-inflammatory mediators.

Many herbal medicines have been reported to be anticonvulsive or antiepileptic based on their effects on behavioral stages and the duration of the latent period [53]. Effects of the drugs were analyzed by comparing the modifications in the stages developed during the process of SE. Two hours of observation of rats after PILO injection gave details of the latent period, the onset, and the behavioral stages of SE. The negative control group which was not treated with any of the drugs developed SE within 27 ± 7 minutes and showed all the stages of the Racine scale. There was no SE in the standard drug-treated group and the rats reached only the second stage of the Racine score and never beyond. All the test groups' readings were in between the negative and positive control groups except in the 2nd dose of MP which showed readings similar to that of standard drug-treated groups. Differences observed in the latent period duration and behavioral stages reached by the animals of the drug-treated and untreated cases were significant ($p < 0.05$).

PILO had a very high impact on WBC, especially on CD4 lymphocytes, IL-1β, and CRP levels when it was injected i.p [50, 54]. Elevated levels of IL-1β may be considered as a hallmark of the effect of PILO-induced TLE models. It may also be considered as a major mediator involved in the dysfunction of BBB [55]. The amount of PILO that reached the brain after systemic injection was considerably low compared to the total amount injected. Also, the direct application of PILO into the brain slices *in vitro* epileptogenic assays showed very poor voltage oscillations. Hence, the effect of PILO in the development of SE was not based on its concentration, but peripheral pro-inflammatory effects and BBB damage developed [56].

In the current analyses, a vast variation in pro-inflammatory parameters was also observed in PILO injected animal models (negative control) during SE when compared to the normal ones (Table 2). Groups that were treated with the test drugs showed comparatively fewer changes than the negative control. Compared to the other groups 2nd dose of MP showed a significantly ($p < 0.05$) good result (Table 1,2 &3). The standard drug-treated group showed no significant variation from the negative control group. This can be explained by the mode of action of the standard drug DZ used. DZ acts on the neurotransmitter receptors in the brain to inhibit seizure development, hence it has less effect on the peripheral factors studied [57].

The confirmation of BBB permeability was done by the quantification of serum albumin in the choroid plexus of the brain. Increased permeability of BBB allows the albumin from the serum to cross the barrier and reach blood vessels in the brain. Except for the animals treated with the second dose of MP and normal ones, an elevated level of serum albumin was observed in the brain tissues of all the studied

groups including the standard drug-treated. As the standard drug has little effect on the peripheral inflammatory factors, BBB permeability was also affected little by the drug. Neurotransmitter levels in the brain tissues (Table 3) also showed that 2nd dose of MP gave better results in reducing seizure activity than DC. MP showed similar activity as that of standard drug. Focal administration of PILO in the hippocampal region was characterized initially by a decrease in GABA and glutamate concentrations followed by their significant increase. Administration of muscarinic receptor antagonists could reduce all these elevations [58]. The current study also showed an elevated glutamate level after PILO administration which was reduced by the treated drugs.

MP, the compound found in the hexane extract of *V. coriaceum* had the highest inhibitory action on trypsin, LOX, and COX *in vitro* [3]. As the enzymes targeted above are important in inflammatory pathways, the potential of MP in the aspect of its anti-inflammation was revealed. *In vivo* models explained in the current study also revealed the compound's potential to regulate inflammatory markers in the blood. BBB permeability is greatly influenced by the elevated inflammatory markers and MP which regulates the inflammatory markers could also regulate BBB permeability. This explains the compounds' role in reducing the signs and symptoms of epilepsy. Not only the brain insults can develop seizures peripheral insults can also affect the brain functioning and in turn, result in seizures. Inhibitory action on peripheral effects developed by PILO administration was found to reduce the SE and then seizure development. The physiological roles of MP in reducing inflammation and other disorders like vasodilation, neuroprotection, immunomodulation, and the anti-apoptotic effect were also reported [59–62]. This also explains the action of MP in reducing inflammatory markers and epilepsy.

The initial *in silico* analysis employed for finding the candidates for the detailed study is suggestive of more prospective candidates might have been left out from the focus of the present study. The chemical profile of the plant under the present study with the specified extracts, together with the existing knowledge of its main components studied, would form a basis for its further, critical analysis by network pharmacology methods towards developing anti-neuroinflammatory protocols. This might pave way for its wider application in the form of extracts or a reconstituted mixture of active compounds present in the natural extracts for desired applications, such as preventive agents against neuro-inflammation-related disorders. However, further studies would be necessary to establish the bio-safety, the non-toxicity, bioavailability, and other biological properties of the compounds and the extracts included in the present study.

Declarations

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Authors' Contributions

The authors declare that all the works stated in the article were done by themselves. VV performed all the experimental works and compiled the data. AJ, MC and SCS helped VV in performing the work. MH, EJV and AS conceived the research program, and guided the work. MH gave final shape to the manuscript. All the authors have read and approved the final manuscript.

Data availability statement

The data used to support the findings of this study are available from the corresponding author upon request.

Competing Interests

The authors declare that they have no competing interests.

Convention on biodiversity

The authors procured permission from the Forest Department, Govt. of Kerala, India to visit and collect the plant sample from the forest areas for research purposes as per Order No. WL 10-47474/2014 dated 1/12/2015 and accordingly procured the sample for the study.

Botanical aspects

The name of the plant is *Viburnum coriaceum* Blume. Plant material was collected from the Nilgiri Hills, India during the month of August. The material was identified and authenticated by Dr. Prabhukumar K. M., Scientist and Head, Plant Systematics and Genetics Division, Centre for Medicinal Plants Research (CMPR), Kottakkal, India and the voucher specimen (CMPR 8670) was deposited at CMPR Raw Drug Museum. Plant materials were dried under shade and powdered. Roots and aerial parts were powdered separately. Roots and aerial parts were extracted with hexane, methanol and water successively. All the extracts were concentrated and stored for further studies.

Ethics approval statement

Al the experiments on animals were carried out according to the guidelines of the Committee for Control and Supervision of Experiments on Animals (CPCSEA), India and ARRIVE (Animal Research: Reporting of In Vivo Experiments) after getting approval from the Internal Animal Ethical Committee of Kannur University, Kerala, India (KULS/IAEC/2017/19).

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Figures

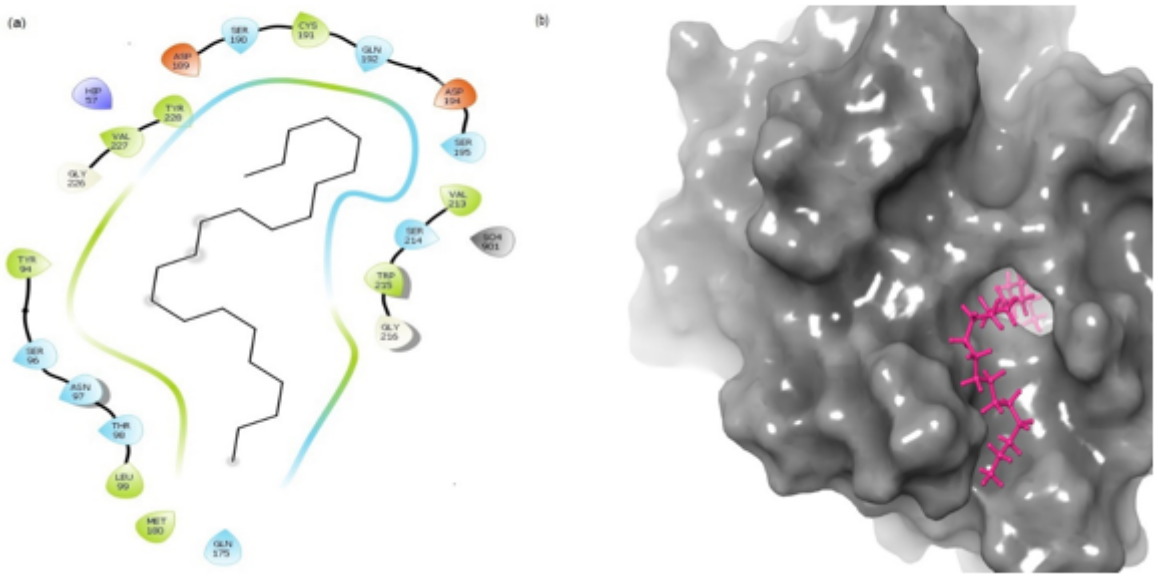

Figure 1

(a) 2D and (b) 3D structures of the interactions of docosane with the enzyme trypsin by the extra precision method of docking

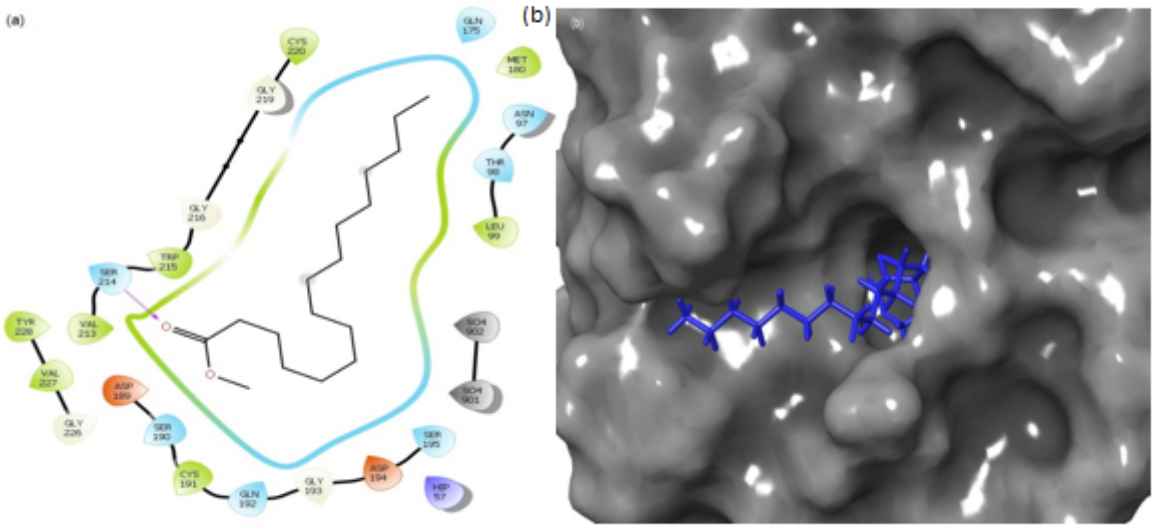

Figure 2

(a) 2D and (b) 3D structures of the interactions of methyl palmitate with the enzyme trypsin by the extra precision method of docking

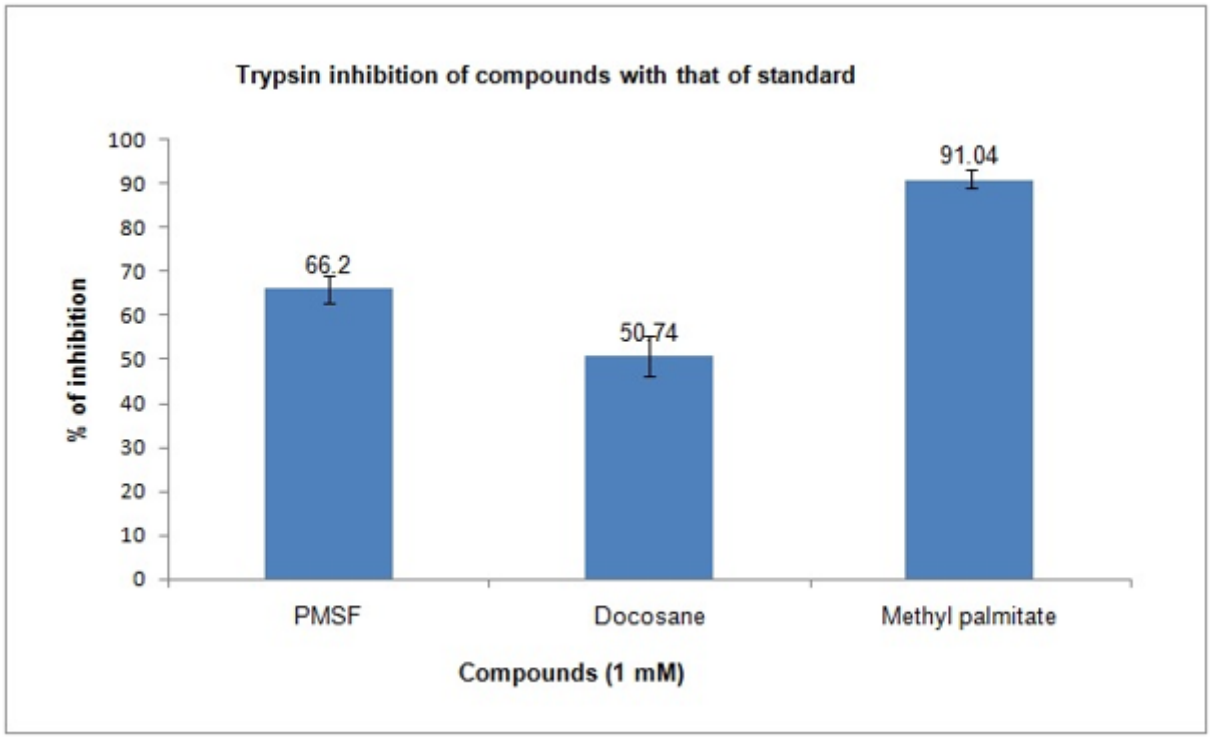

Figure 3

Percentage of inhibition by the compounds and standard on trypsin

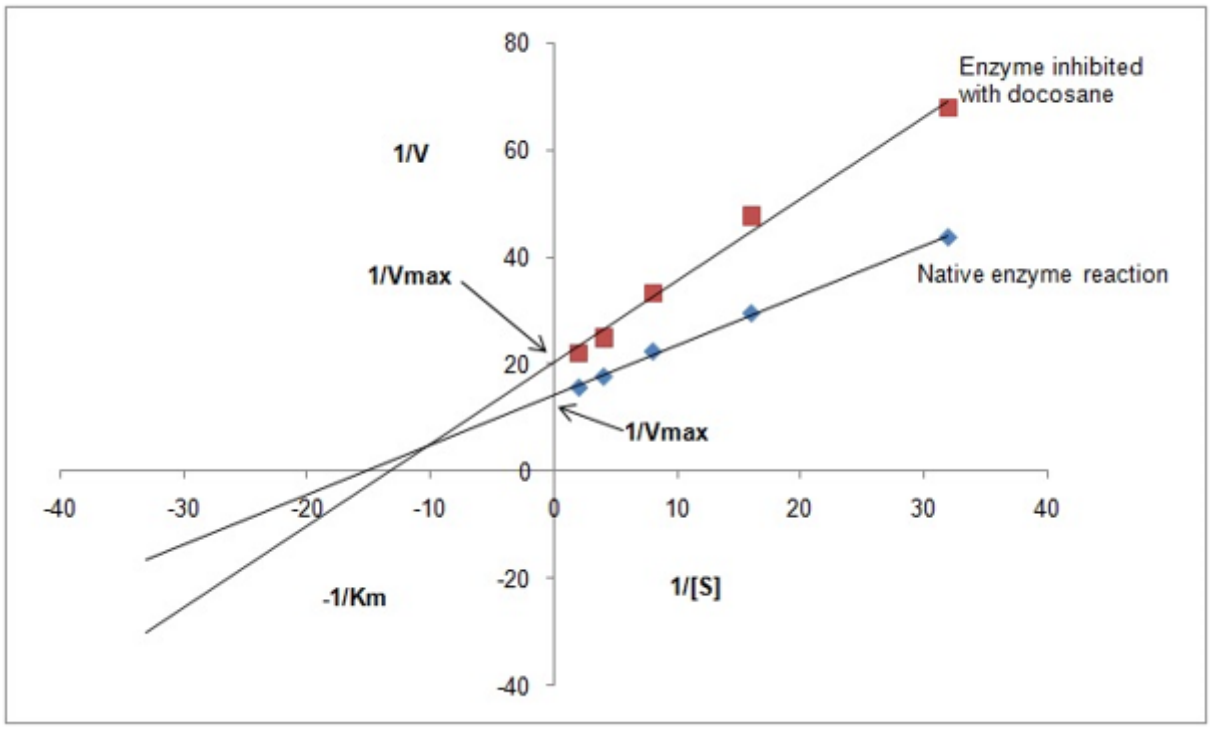

Figure 4

Lineweaver-Burk plot of trypsin inhibition by docosane

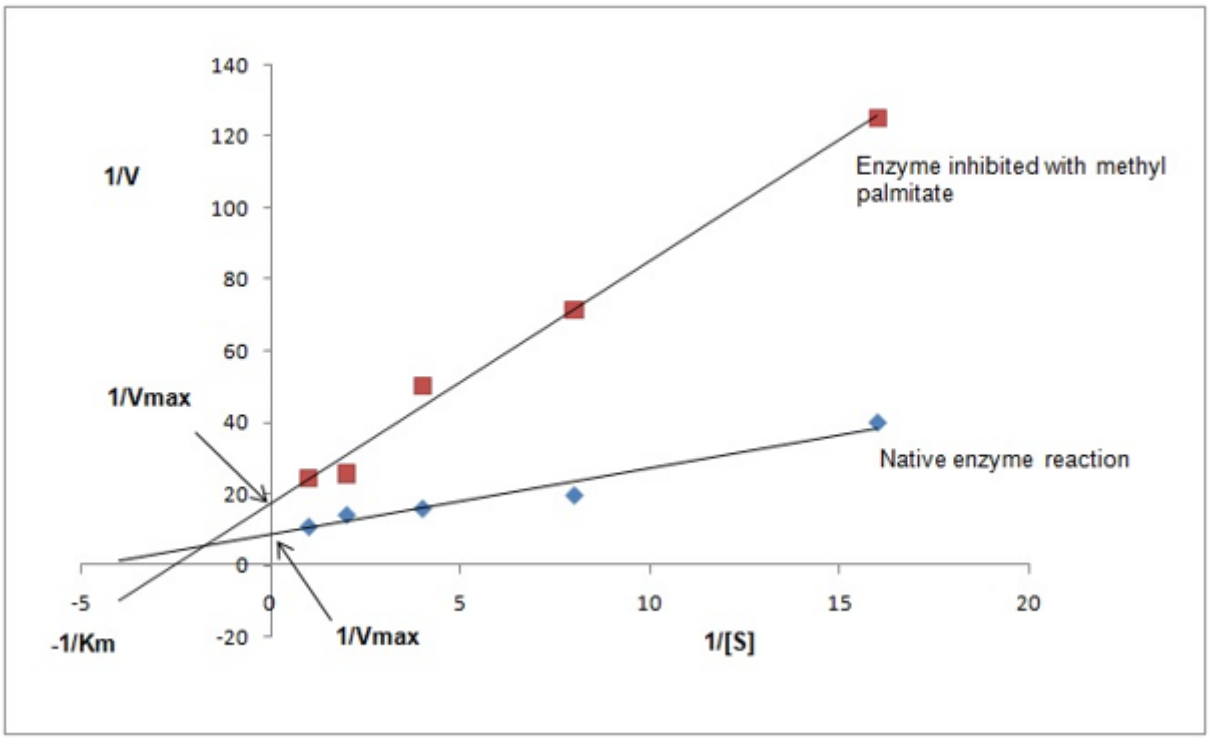

Figure 5

Lineweaver-Burk plot of trypsin inhibition by methyl palmitate

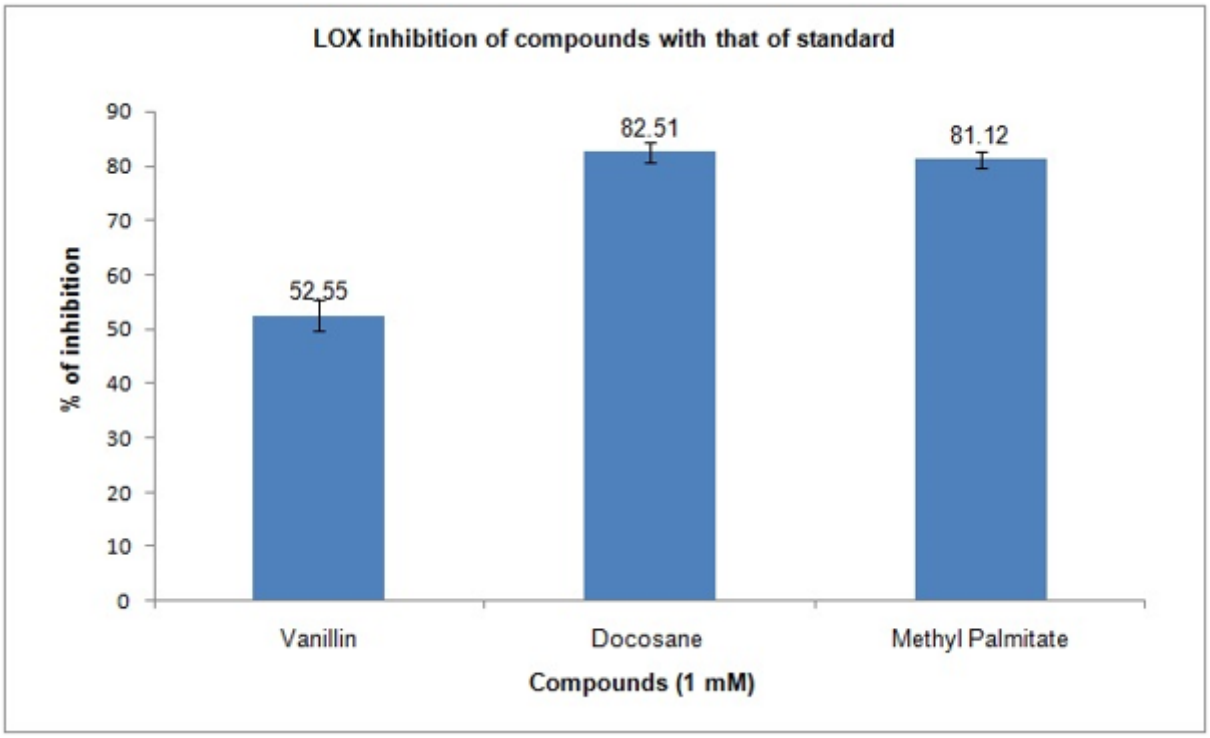

Figure 6

LOX inhibitory effect of the compounds and the standard

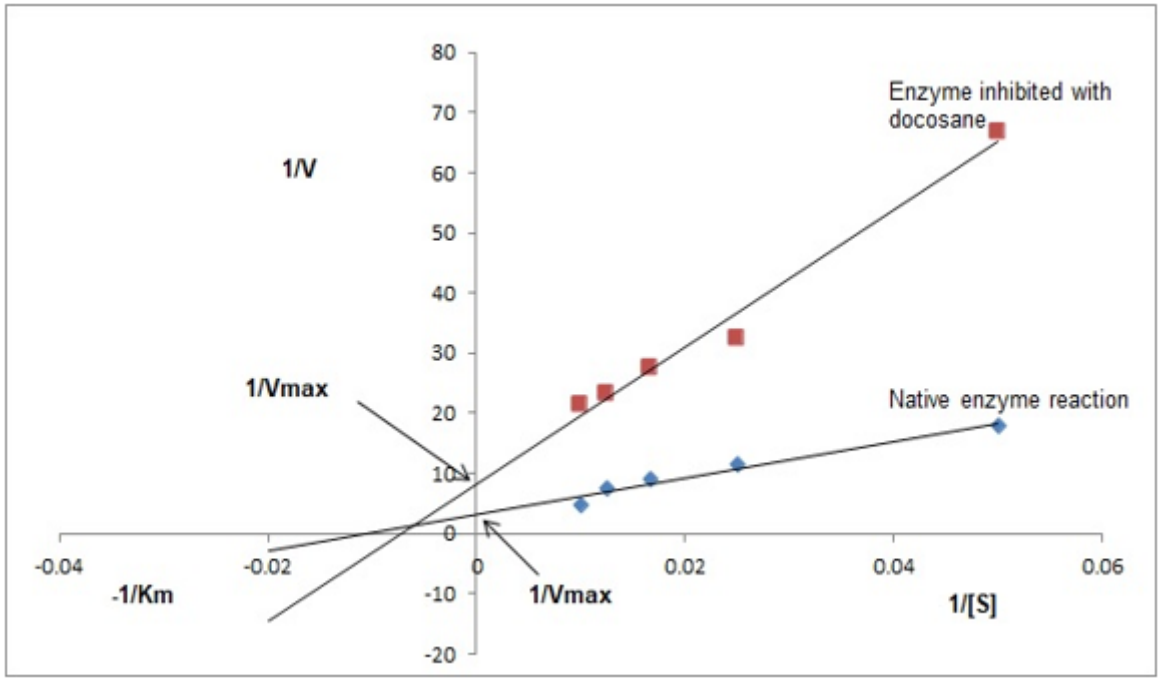

Figure 7

Lineweaver-Burk plot of LOX inhibition by docosane

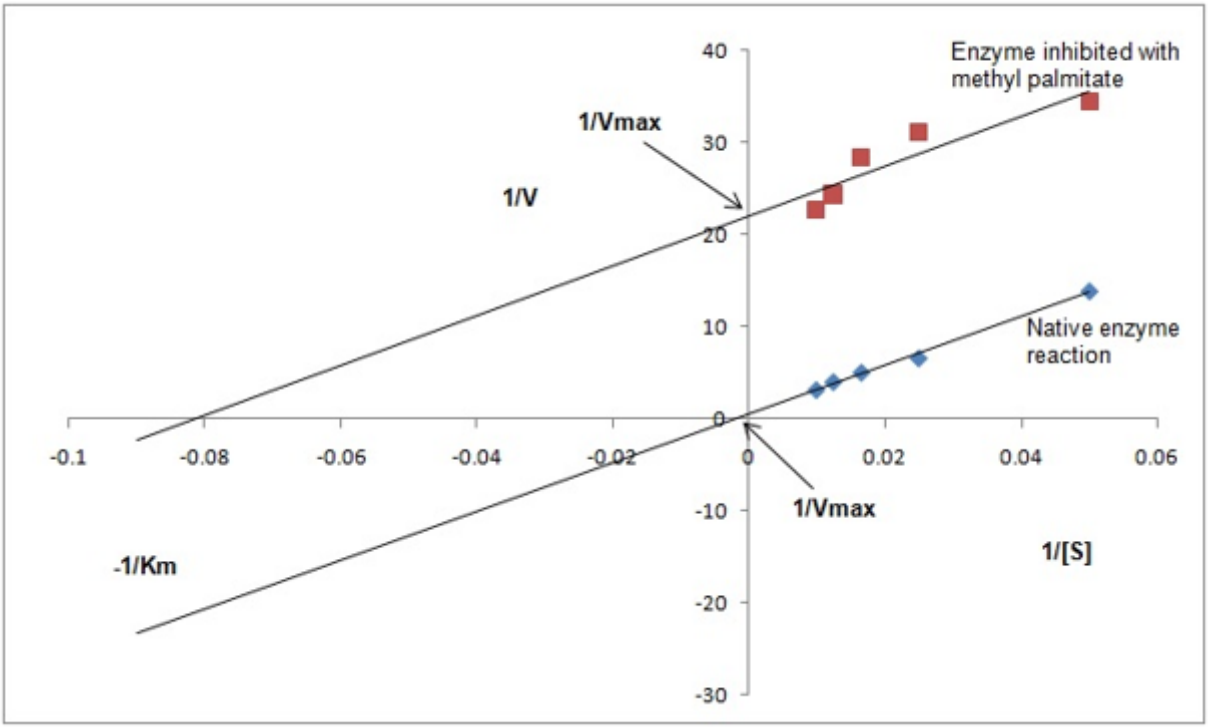

Figure 8

Lineweaver-Burk plot of LOX inhibition by methyl palmitate

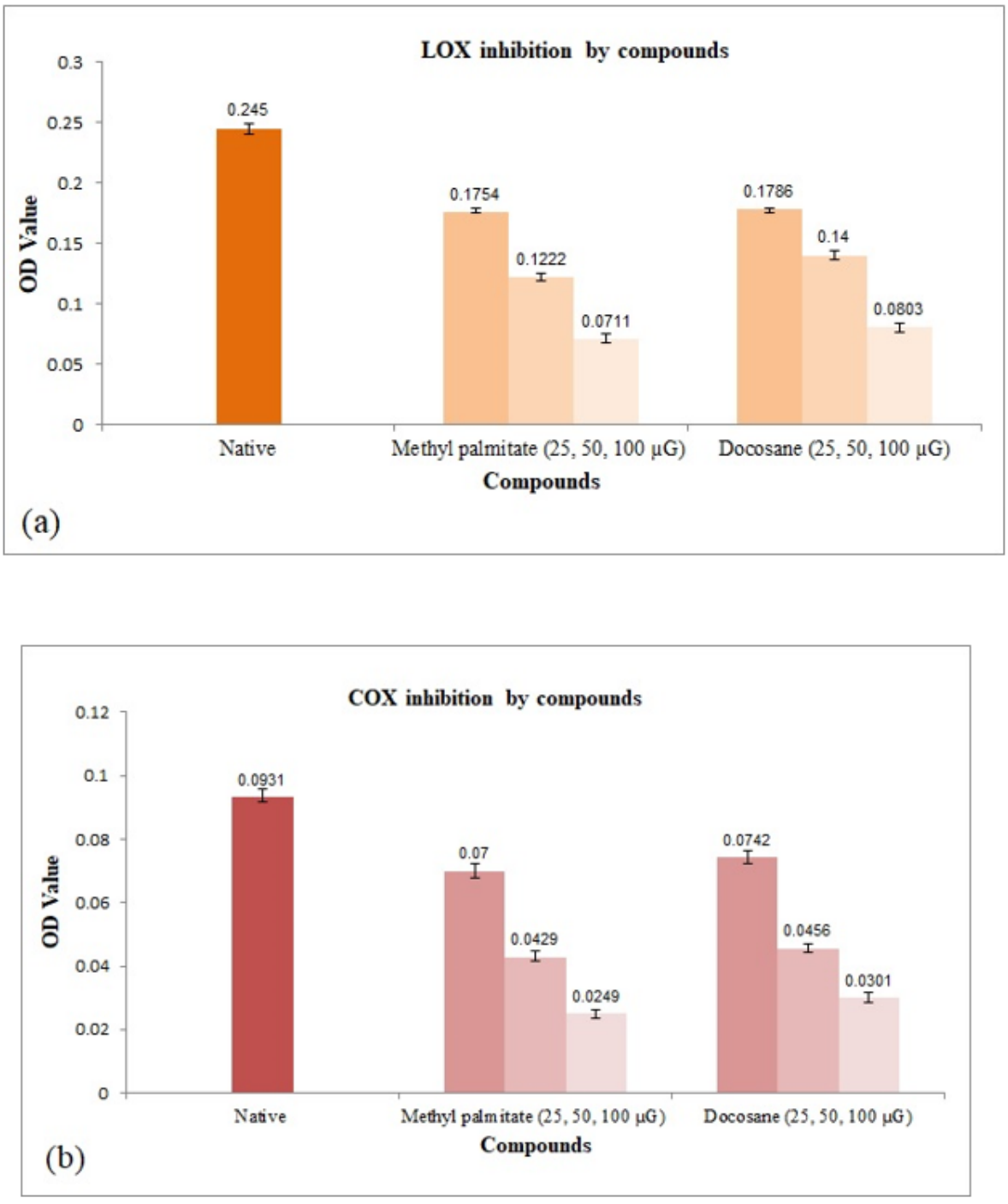

Figure 9

(a) LOX and (b) COX inhibitory activities of methyl palmitate and docosane on cell extracts

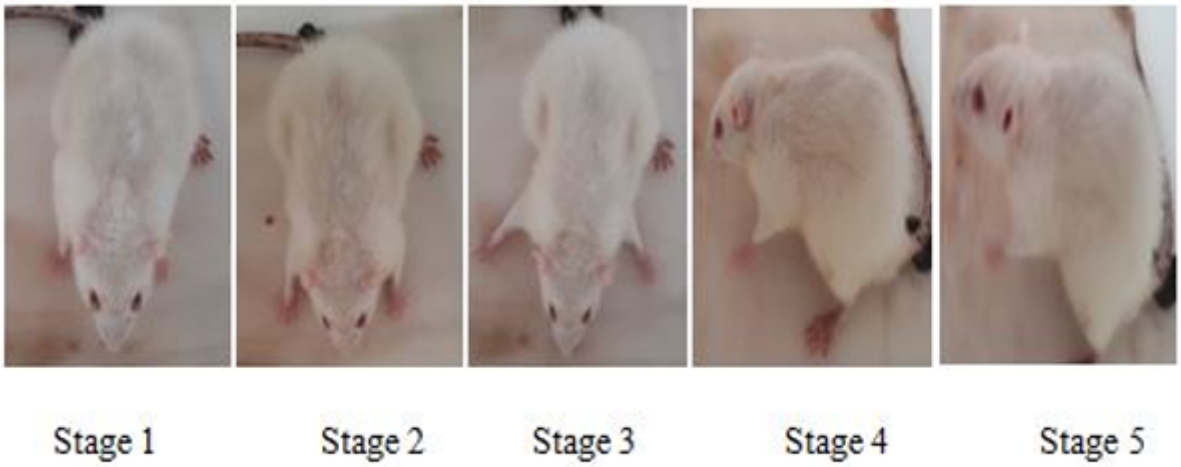

Figure 10

Behavioral stages of the rat at pilocarpine model of temporal lobe epilepsy

Supplementary Files

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- SupplementaryInformation.docx