



## RESEARCH ARTICLE

# Antinociceptive and sedative activity of *Vernonia patula* and predictive interactions of its phenolic compounds with the cannabinoid type 1 receptor

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When tested in the acetic acid-induced writhing and formalin-induced paw-licking tests, the ethanol extract of *Vernonia patula* (VP) aerial parts showed significant antinociceptive activity. In neuropharmacological tests, it also significantly delayed the onset of sleep, increased the duration of sleeping time, and significantly reduced the locomotor activity and exploratory behaviour of mice. Five phenolic compounds, namely gallic acid, vanillic acid, caffeic acid, quercetin and kaempferol, were detected in VP following HPLC-DAD analysis. The presence of these phenolic compounds in VP provides some support for the observed antinociceptive and sedative effects. A computational study was performed to predict the binding affinity of gallic acid, vanillic acid, caffeic acid, quercetin and kaempferol towards the cannabinoid type 1 (CB1) receptor. Caffeic and vanillic acid showed the highest probable ligand efficiency indices towards the CB1 target. Vanillic acid displayed the best blood-brain barrier penetration prediction score. These findings provide some evidence for the traditional use of VP to treat pain.

**KEYWORDS**

antinociceptive activity, cannabinoid receptor 1 (CB1), molecular docking, *Vernonia patula*

## 1 | INTRODUCTION

*Vernonia patula* (Dryand.) Merr. (Asteraceae), also known as purple fleabane, is an annual weed widely distributed throughout Bangladesh. The plant is used traditionally as an aphrodisiac, and for malaria, respiratory tract disorders, colds, fevers and convulsions (Khare, 2007; Mollik et al., 2010). The leaves are reputed to be beneficial in treating pain (Jahan et al., 2015). Previous chemical studies of the aerial parts of *V. patula* have revealed the presence of phenolic compounds, flavonoids and terpenoids (Herrera, Chanco, & Sison, 1980; Liang & Min, 2003; Lin & Wang, 2002). We have already reported that the aerial parts of *V. patula* possess in vitro anti-oxidant

and in vivo antiinflammatory activity (Hira et al., 2013). Other species of *Vernonia*, including *Vernonia amygdalina*, *Vernonia cinerea* and *Vernonia polyanthes*, have demonstrated antinociceptive and sedative properties (Iwalewa, Iwalewa, & Adeboye, 2003; Muir, 1981; Temponi Vdos et al., 2012). To the best of our knowledge the antinociceptive and sedative activity of *V. patula* has never been investigated.

The cannabinoid receptors (CB) are members of the superfamily of G protein-coupled receptors. Two types have been reported; the CBR type-1 (CB1) and type-2 (CB2). The CB1 receptors, which regulate pain, movement and neurotic parameters, are found in some peripheral tissues (Roche et al., 2006; Wright et al., 2005), but mostly in the central nervous system (CNS) (Cravatt & Lichtman, 2004;

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Giuffrida et al., 1999; Hampson & Deadwyler, 1999). Here, we analysed the aerial parts *V. patula* for the presence of phenolic compounds, and tested its ethanol extract for antinociceptive and sedative activity in mice. We also conducted a molecular docking study to predict the interactions between the phenolic compounds detected in *V. patula* (VP) and the CB1 receptor in an effort to establish a putative mode of action for the phenolic compounds present in this plant.

## 2 | MATERIALS AND METHODS

### 2.1 | Chemicals and drugs

Acetic acid, carrageenan, diclofenac sodium salt, histamine phosphate, indomethacin, pentobarbital, diazepam, the synthetic tetrahydrocannabinol (THC) dronabinol, morphine sulphate and the opioid receptor antagonist naloxone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethanol and formalin were purchased from Merck, Germany. Aluminium chloride, potassium acetate, sodium carbonate and Tween 80 were used as analytical grade reagents and bought from Merck, India Ltd.

### 2.2 | Plant collection and extraction

The aerial parts of *V. patula* (VP) were collected from Chittagong (Bangladesh) in 2015. The plant material was identified by M. A. Ali at the Bangladesh National Herbarium in Dhaka where a voucher specimen (DACB: 35107) is kept for future reference. The dried powdered aerial parts (500 g) were macerated in EtOH (1.5 L) for 5 days at  $25 \pm 2^\circ\text{C}$ , with occasional shaking, and the resulting filtrate was concentrated under reduced pressure to yield the extract (66 g, 13.2% yield).

### 2.3 | HPLC-DAD analysis

Phenolic compounds in VP aerial parts were detected using a Dionex UltiMate 3000 LC system and Acclaim® C18 ( $4.6 \times 250$  mm;  $5 \mu\text{m}$ ) column (Sumi et al., 2016). A mixed solvent system of acetonitrile (solvent A), acetic acid solution at pH 3.0 (solvent B) and methanol (solvent C) was used as a mobile phase with the following gradient elution (0 min: 5% A/95% B; 10 min: 10% A/80% B/10% C; 20 min: 20% A/60% B/20% C and 30 min: 100% A). The extract ( $20 \mu\text{L}$ ) was injected at a flow rate of 1 mL/min, which was maintained constant throughout the analysis. The diode array detector (DAD) was set from  $\lambda = 200$  to 700 nm. Specific wavelengths for the detection of phenolic compounds were set up as follows:  $\lambda = 280$  nm for 18.0 min (caffeic acid, catechin, vanillic acid, epicatechin and gallic acid),  $\lambda = 320$  nm for the next 6 min (ellagic acid, *p*-coumaric acid and rutin), and finally  $\lambda = 380$  nm for the rest of the analysis (myricetin, kaempferol and quercetin). A stock solution ( $100 \mu\text{g/mL}$ ) of each of the aforementioned phenolic compounds was prepared and further diluted to  $20 \mu\text{g/mL}$ , except for caffeic acid ( $8 \mu\text{g/mL}$ ) and quercetin ( $6 \mu\text{g/mL}$ ). A solution of VP ( $5 \text{ mg/mL}$ ) was

prepared in ethanol. Spiked samples were also prepared by mixing the VP solution with each of the phenolic standards. All solutions were filtered through a  $0.20 \mu\text{m}$  nylon syringe filter (Sartorius, Germany) prior to the HPLC analysis. The phenolic compounds present in VP were identified by comparing their retention times and absorbance profiles with the standards. Calibration curves were prepared with serial dilutions of each phenolic standard in methanol, ranging from  $1.25$  to  $20 \mu\text{g/mL}$  (gallic acid, myricetin, vanillic acid, epicatechin, *p*-coumaric acid, kaempferol and ellagic acid),  $0.5$ – $8.0 \mu\text{g/mL}$  for caffeic acid, catechin and rutin, and  $0.375$ – $6.0 \mu\text{g/mL}$  for quercetin. Quantification of the peaks was performed with  $R^2 > 0.995$ . Data were obtained as means  $\pm$  SD of three independent analyses (Table S1, Figure S1).

### 2.4 | Test animals

Swiss albino mice ( $20$ – $25$  g) of both sexes were collected from the animal research branch of the International Center for Diarrheal Disease and Research in Bangladesh (ICDDR, B) and kept under standard laboratory conditions ( $25 \pm 2^\circ\text{C}$  and 12/12 h light/dark cycle). The animals were fed with standard food and water ad libitum during the acclimatisation period. Before any experiments, the animals were kept fasted overnight. All animals were handled according to the ethical principles and guidelines for experiments on animals (Swiss Academy of Medical Sciences and Swiss Academy of Sciences, 2005) and approved by the BCSIR ethics committee (BCSIR/IAEC/01/13-14).

### 2.5 | Antinociceptive tests

#### 2.5.1 | Acetic acid-induced writhing test

This was used to evaluate peripheral antinociceptive behaviour (Whittle, 1964). Briefly, the mice were divided into five groups ( $n = 5$ ). The negative control group was treated with 1% Tween 80 in normal saline ( $10 \text{ mL/kg}$ , p.o.). Diclofenac sodium ( $25 \text{ mg/kg}$ ) was administered intraperitoneally to the positive control group. Test groups were treated with the VP extract ( $100$ ,  $200$  and  $400 \text{ mg/kg}$  respectively, p.o.) 30 min before intraperitoneal injection of 0.7% acetic acid. After a 5 min interval, the constriction of the abdomen and extension of hind legs (writhing) of the animals was determined for 10 min. The ability of the VP extract and diclofenac to reduce the number of writhing was calculated using Equation (S1.1).

#### 2.5.2 | Formalin-induced paw licking test

This was performed using a previously published protocol (Dubuisson & Dennis, 1977). Briefly, overnight-fasted mice were distributed into six groups ( $n = 5$ ). The negative control group was treated with 1% Tween 80 in normal saline ( $10 \text{ mL/kg}$ , p.o.). Diclofenac sodium ( $25 \text{ mg/kg}$ ) and morphine ( $5 \text{ mg/kg}$ ) were administered subcutaneously (s.c.) in the positive control group as phase

1 and phase 2 positive controls, respectively. The VP extract (100, 200 and 400 mg/kg, p.o.) was administered to the test group. A 1% formalin solution in saline (20  $\mu$ L) was then injected s.c. to all groups. After the formalin injection, the licking or biting time of the injured paw was recorded as a nociceptive response, considering the first 5 min as phase-1 and the next 15–30 min as phase-2, respectively (Silva, Martins, Matheus, Leitao, & Fernandes, 2005).

### 2.5.3 | Hot plate test

The mice were placed on a hot metal plate ( $50 \pm 0.5^\circ\text{C}$ ). The time period between the placement of the animal on the hot surface and its reaction—by lifting or licking its paws—to avoid thermal pain was recorded as the response latency (Eddy & Leimbach, 1953). Twenty seconds were used as a cut-off period to avoid any tissue damage of the paws. Mice were pre-treated with 1% Tween 80 in normal saline (10 mL/kg, p.o.) and morphine (5 mg/kg, i.p.) in the negative and positive control group, respectively. The VP extract (100, 200 and 400 mg/kg, p.o.) was administered to the test groups. The response time was divided into different intervals, that is, 0, 30, 60, 90 and 120 min. On the other hand, to identify the central analgesic impact of VP, naloxone (2 mg/kg, i.p.) was injected to five new groups 15 min prior to the administration of either morphine (10 mg/kg, i.p.) or the VP extract (100, 200 and 400 mg/kg, p.o.). The antinociceptive activity was evaluated based on increasing the latency time to the thermal stimulus compared to the negative control group. Equation (S1.2) was used to calculate the percentage of the maximal possible effect (% MPE).

### 2.5.4 | Tail immersion test

This was performed according to a previously published method (Silva et al., 2005). Five groups of mice ( $n = 5$ ) were pretreated with VP (100, 200 and 400 mg/kg, p.o.) and with 1% Tween 80 in normal saline (10 mL/kg, p.o.) and morphine (5 mg/kg, i.p.) for the negative and positive control groups, respectively. Their tails were immersed in warm water ( $55 \pm 1^\circ\text{C}$ ). The latency between tail submersion and deflection of the tail was recorded. Pre-treatment latency was recorded at 30, 60, 90 and 120 min. In a similar experiment, another five groups of mice ( $n = 5$ ) were used and, to verify central analgesic activity, were administered naloxone (2 mg/kg, i.p.). The antinociceptive activity was evaluated based on the latency period of the tail withdrawal response. Equation (S1.2) was used to calculate the % MPE.

## 2.6 | Neuropharmacological tests

### 2.6.1 | Pentobarbital-induced hypnosis

This was performed according to a previously published method with some modifications (Estrada-Reyes, Martinez-Vazquez, Gallegos-Solis, Heinze, & Moreno, 2010). The mice were divided into five groups ( $n = 5$ ). Three

groups received the VP extract (100, 200 and 400 mg/kg, p.o.) 30 min before the administration of pentobarbital (40 mg/kg i.p.). The negative and the positive control group received 1% Tween 80 in normal saline (10 mL/kg, p.o.) and the standard drug diazepam (1 mg/kg, p.o.), respectively, 30 min before the administration of pentobarbital (40 mg/kg i.p.). The time period between the pentobarbital administration and the onset of sleep (i.e., when the animals lost their righting reflex) as well as the duration of sleep (i.e., time elapsed between the loss and recovery of the righting reflex) were recorded to evaluate the total hypnotic effect of VP in mice.

### 2.6.2 | Open field test

This assessed both locomotor and emotional activity in mice using an open field apparatus (Shilpi et al., 2006). The mice were placed in a box (100 cm  $\times$  100 cm  $\times$  40 cm in height) with a floor divided with painted lines forming 25 (20 cm  $\times$  20 cm) squares. The mice were divided into five groups ( $n = 7$ ). Three groups received the VP extract (100, 200 and 400 mg/kg, p.o.) whilst the negative and the positive control group received 1% Tween 80 in normal saline (10 mL/kg, p.o.) and diazepam (1 mg/kg, i.p.), respectively. Locomotor activity was recorded as the number of crossed lines visited by each mouse for 3 min at 0, 30, 60, 90, 120, 180, and 240 min after oral administration of the test drugs. Different groups were used for different evaluation times. The percentage inhibition of movements (% MI) was calculated using Equation (S1.3).

### 2.6.3 | Hole cross test

This test was performed using a cage (30 cm  $\times$  20 cm  $\times$  14 cm in height) with a steel partition containing a hole (3 cm in diameter and 7.5 cm in height) in the centre (Takagi, Watanabe, Saito, 1971). The number of mice which passed from one chamber to the other through the hole was calculated after administering the test drugs for 3 min at 0, 30, 60, 90, 120, 180 and 240 min during the study period. Different groups were used for different evaluation times. The % MI was calculated using Equation (S1.3).

## 2.7 | Statistical analysis

One-way or two-way analysis of variance (ANOVA) followed by Dunnett's test was used when comparing the test samples with the negative control. Variances between different groups were measured to a significance at a near of  $p < .05$ . All statistical analyses were performed using the SPSS software v.11.5.

## 2.8 | Computational study

### 2.8.1 | Ligands optimisation

Gallic acid, vanillic acid, caffeic acid, quercetin and kaempferol were used as the ligands for the in silico work. Their three dimensional

structures were obtained from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) and converted to protein data bank (PDB) formats using GaussView v.5.0 (<https://gaussian.com/gaussview6/>).

## 2.8.2 | Protein optimisation

The crystal structure of the cannabinoid receptor 1 (CB1) was retrieved from the PDB database (PDB ID: 5U09). Energy minimisation was performed using Swiss-PDB Viewer v. 4.1.0 (Guex & Peitsch, 1997). The heteroatoms and water molecules were removed from the crystal structure using PyMOL Molecular Graphics System v. 1.3 (<https://pymol.org>) prior to docking. The protein and ligand structures were saved in the PDBQT format.

## 2.8.3 | Determination of ligand-protein binding affinity and non-bonding interactions

The active binding pocket of CB1 was predicted by CASTp (v. 3.0) (Dundas et al., 2006). The protein (ID- J\_5C2DB3AA670AB) showed the highest pocket area and volume at 652.65 Å<sup>2</sup> and 331.44 Å<sup>3</sup>, respectively. This predicted binding pocket was used for the generation of the grid box to dock the triterpenoids against CB1. The centre of the grid box was set at 12.5230, 7.2495 and 17.7743 Å and the box size was set at 81.5, 81.5 and 81.5 Å in x, y and z directions, respectively. AutoDock Vina (v. 1.1.2) was used to perform the molecular docking study (Trott & Olson, 2010). The docked pose of the lowest binding free energy conformer (showing the highest probable binding affinity) with CB1 was analysed using PyMOL Molecular Graphics System v. 1.3 (<https://pymol.org>), Drug Discovery Studio v. 4.1 (<https://www.3dsbiovia.com>), and LigPlot<sup>+</sup> v. 1.4.5 (<https://www.ebi.ac.uk/thornton-srv/software/LigPlus/>). Ligand efficiency (LE) indices were calculated by dividing the predicted free energy of binding ( $-\Delta G$ ) by the molecular weight (MW) for each compound (García-Sosa, Hetényi, & Maran, 2010).

## 2.8.4 | Prediction of pharmacokinetic and drug-likeness properties

The pharmacokinetic and drug-likeness properties of (1–5) were predicted using the online databases admetSAR (Yang et al., 2019) and SwissADME (Daina, Michielin, & Zoete, 2017), respectively. The Moriguchi LogP (MLogP) calculation, was used as an alternative to LogP (Sterling, Kogler, Anderson, & Brodowicz, 2014).

## 3 | RESULTS

### 3.1 | Detection of phenolic compounds

Analysis of the ethanol extract of VP by HPLC-DAD revealed the presence of high amounts of caffeic acid and quercetin (123.75 and

96.35 mg/100 g dry extract, respectively). The extract also contained kaempferol, gallic acid, and vanillic acid (28.28, 17.82 and 8.91 mg/100 g of dry extract, respectively) (Table 1, Figure S2).

### 3.2 | Acetic acid-induced writhing test

VP demonstrated a significant dose-dependent decrease in the number of writhing responses. At 400 mg/kg, VP exerted a writhing inhibitory effect of 65.22% that was comparable to diclofenac (78.74%) (Table 2).

### 3.3 | Formalin-induced paw licking test

In this assay, morphine inhibited the number of licking responses in both phase-1 (68.43%) and phase-2 (98.48%). The standard drug diclofenac inhibited the number of licking responses only in the phase-2 (97.69%). VP showed a statistically significant substantial inhibition of licking responses at all doses in phase-1 and phase-2. At a dose of 400 mg/kg, it showed a high reduction of licking response of 55.20% in phase-1 and 68.20% in phase-2 (Table 3).

### 3.4 | Hot plate test

VP demonstrated a dose-dependent central antinociceptive effect with a maximum increase in the reaction time to the thermal stimulus at the dose of 400 mg/kg after 90 min (6.63 s) that was statistically significant compared to the negative control ( $p < .001$ ). At that same dose, VP also showed a reaction time (5.15 s) that was higher than morphine. When naloxone was administered together either with VP or with morphine, the analgesic effect was overall reversed, but no statistical significance was observed (Table 4).

### 3.5 | Tail immersion test

All tested doses of VP (100, 200 and 400 mg/kg) significantly increased the latency period to the hot water-induced thermal stimulus, with the highest increase (7.11 s) seen after 45 min, and statistically significant

**TABLE 1** HPLC-DAD detection and quantification of phenolic compounds in VP aerial parts

| Compound          | Content (mg/100 g of dry extract) | % RSD |
|-------------------|-----------------------------------|-------|
| Caffeic acid (1)  | 123.75                            | 1.97  |
| Quercetin (2)     | 96.35                             | 1.88  |
| Kaempferol (3)    | 28.28                             | 0.98  |
| Gallic acid (4)   | 17.82                             | 0.63  |
| Vanillic acid (5) | 8.91                              | 0.21  |

**TABLE 2** Effects of VP and diclofenac in the acetic-acid induced writhing test<sup>a</sup>

| Treatment         | Dose (mg/kg)    | Number of writhes (% writhing) | % Writhing inhibition |
|-------------------|-----------------|--------------------------------|-----------------------|
| Vehicle (control) | 10 <sup>b</sup> | 20.70 ± 1.16 (100)             | —                     |
| Diclofenac        | 25              | 4.40 ± 0.82 (21.26)**          | 78.74                 |
| VP                | 100             | 9.40 ± 0.81(45.41)**           | 54.59                 |
| VP                | 200             | 8.20 ± 0.66(39.61)**           | 60.39                 |
| VP                | 400             | 7.20 ± 0.49 (34.78)**          | 65.22                 |

Abbreviation: VP = *Vernonia patula* ethanol extract.<sup>a</sup>Values are mean ± SEM (n = 5).<sup>b</sup>In mL/kg.

\*\*p &lt; .01 vs control (one-way ANOVA followed by Dunnett's test).

**TABLE 3** Effects of VP, morphine and diclofenac in the formalin-induced paw licking test<sup>a</sup>

| Treatment         | Dose (mg/kg)    | Licking of the hind paw |              |                     |              |
|-------------------|-----------------|-------------------------|--------------|---------------------|--------------|
|                   |                 | Phase-1 (0–5 min)       | % Inhibition | Phase-2 (15–30 min) | % Inhibition |
| Vehicle (control) | 10 <sup>b</sup> | 113.40 ± 4.50           | —            | 121.40 ± 3.96       | —            |
| Morphine          | 5               | 35.80 ± 2.40****        | 68.43        | 1.76 ± 0.64****     | 98.55        |
| Diclofenac        | 10              | 68.20 ± 2.41****        | 39.86        | 2.80 ± 0.86****     | 97.69        |
| VP                | 100             | 73.20 ± 3.61****        | 35.44        | 70.20 ± 3.31****    | 42.17        |
| VP                | 200             | 62.40 ± 2.33****        | 44.97        | 51.40 ± 2.75****    | 57.66        |
| VP                | 400             | 50.80 ± 2.27****        | 55.20        | 38.60 ± 1.86****    | 68.20        |

Abbreviation: VP = *Vernonia patula* ethanol extract.<sup>a</sup>Values are mean ± SEM (n = 5).<sup>b</sup>In mL/kg.

\*\*\*\*p &lt; .0001 vs control (two-way ANOVA followed by Dunnett's test).

**TABLE 4** Effects of VP, morphine and reversal effect of naloxone in the hot plate test<sup>a</sup>

| Treatment         | Dose (mg/kg)    | Latency period (s) (% MPE) |                       |                       |                       |                        |                       |
|-------------------|-----------------|----------------------------|-----------------------|-----------------------|-----------------------|------------------------|-----------------------|
|                   |                 | 0 min                      | 30 min                | 45 min                | 60 min                | 90 min                 | 120 min               |
| Vehicle (control) | 10 <sup>b</sup> | 2.08 ± 0.20                | 2.48 ± 0.15           | 2.49 ± 0.19           | 2.55 ± 0.31           | 2.39 ± 0.28            | 2.73 ± 0.31           |
| Morphine          | 5               | 2.46 ± 0.28                | 9.69 ± 0.55(41.22)*** | 9.77 ± 0.34(41.65)*** | 9.98 ± 0.68(42.91)*** | 8.60 ± 0.54(35.02)***  | 4.39 ± 0.28(11.01)*** |
| VP                | 100             | 2.32 ± 0.22                | 2.59 ± 0.07 (1.51)    | 3.52 ± 0.53 (6.81)*   | 4.05 ± 0.61 (9.79)*   | 4.29 ± 0.47 (11.14)**  | 4.03 ± 0.53 (9.67)*   |
| VP                | 200             | 2.31 ± 0.21                | 3.01 ± 0.28 (3.95)*   | 3.81 ± 0.38 (8.48)**  | 4.59 ± 0.46 (12.89)** | 5.37 ± 0.51 (17.30)*** | 3.99 ± 0.55 (9.49)*   |
| VP                | 400             | 2.43 ± 0.11                | 3.93 ± 0.44 (8.51)**  | 5.57 ± 0.25(17.92)*** | 5.63 ± 0.37(18.22)*** | 6.63 ± 0.36(23.92)***  | 5.15 ± 0.43(15.49)*** |
| NLX               | 2               | 2.73 ± 0.32                | 2.69 ± 0.43           | 2.75 ± 0.31           | 2.47 ± 0.25           | 2.58 ± 0.34            | 1.89 ± 0.23           |
| NLX + morphine    | 2 + 5           | 2.85 ± 0.29                | 2.58 ± 0.42 (0.27)    | 2.82 ± 0.34 (0.03)    | 2.91 ± 0.33 (−0.06)   | 2.57 ± 0.18 (0.28)     | 2.54 ± 0.30 (0.31)    |
| NLX + VP          | 2 + 100         | 3.08 ± 0.34                | 2.25 ± 0.19 (0.33)    | 2.35 ± 0.43 (0.47)    | 2.88 ± 0.40 (0.03)    | 2.65 ± 0.69 (−0.08)    | 2.65 ± 0.37 (−0.11)   |
| NLX + VP          | 2 + 200         | 2.43 ± 0.28                | 2.42 ± 0.26 (0.16)    | 2.05 ± 0.28 (0.77)    | 2.49 ± 0.41 (0.42)    | 2.51 ± 0.77 (0.06)     | 2.21 ± 0.22 (0.33)    |
| NLX + VP          | 2 + 400         | 2.53 ± 0.29                | 2.89 ± 0.24 (−0.31)   | 3.17 ± 0.39 (−0.35)   | 3.06 ± 0.57 (−0.15)   | 2.87 ± 0.51 (−0.3)     | 2.15 ± 0.19 (0.39)    |

Abbreviations: NLX, Naloxone; VP, *Vernonia patula* ethanol extract.<sup>a</sup>Values are mean ± SEM (n = 5).<sup>b</sup>In mL/kg.

\*p &lt; .05, \*\*p &lt; .01, \*\*\*p &lt; .001 vs control (two-way ANOVA followed by Dunnett's test).

compared to the negative control (p < .05). At all doses, VP demonstrated a central antinociceptive effect with a maximum increase in the reaction time after 120 min (5.05, 5.52 and 5.15 s, respectively) that was

higher than morphine. When naloxone was administered together either with VP or with morphine, the analgesic effect was overall reversed, but no statistical significance was observed (Table 5).

**TABLE 5** Effects of VP, morphine and reversal effect of naloxone in the tail immersion plate test<sup>a</sup>

| Treatment         | Dose (mg/kg)    | Latency period (s) (% MPE) |                      |                       |                       |                       |                      |
|-------------------|-----------------|----------------------------|----------------------|-----------------------|-----------------------|-----------------------|----------------------|
|                   |                 | 0 min                      | 30 min               | 45 min                | 60 min                | 90 min                | 120 min              |
| Vehicle (control) | 10 <sup>b</sup> | 2.35 ± 0.23                | 2.41 ± 0.24          | 2.59 ± 0.16           | 2.33 ± 0.27           | 2.41 ± 0.38           | 2.54 ± 0.35          |
| Morphine          | 5               | 3.04 ± 0.26                | 8.41 ± 0.41(31.66)*  | 12.08 ± 0.73 (53.33)* | 11.83 ± 0.80 (51.80)* | 11.15 ± 0.36 (47.79)* | 4.79 ± 0.64 (10.37)  |
| VP                | 100             | 2.85 ± 0.17                | 5.15 ± 0.29 (13.41)* | 5.56 ± 0.42 (15.78)*  | 5.79 ± 0.30 (17.17)*  | 6.06 ± 0.43 (18.96)*  | 5.05 ± 0.44 (12.89)* |
| VP                | 200             | 2.94 ± 0.12                | 5.83 ± 0.31 (16.95)* | 5.97 ± 0.47 (17.77)*  | 6.28 ± 0.54 (19.57)*  | 6.22 ± 0.58 (19.20)*  | 5.52 ± 0.43 (15.14)* |
| VP                | 400             | 3.08 ± 0.25                | 6.99 ± 0.54 (23.12)* | 7.11 ± 0.52 (23.83)*  | 6.65 ± 0.60 (21.13)*  | 6.64 ± 0.38(21.02)*   | 5.62 ± 0.46(15.03)*  |
| NLX               | 2               | 2.92 ± 0.30                | 2.94 ± 0.26          | 2.88 ± 0.27           | 2.82 ± 0.28           | 2.96 ± 0.27           | 2.66 ± 0.22          |
| NLX + morphine    | 2 + 5           | 2.70 ± 0.23                | 2.98 ± 0.33 (-0.28)  | 3.08 ± 0.22 (-0.38)   | 2.98 ± 0.29 (-0.28)   | 3.14 ± 0.26 (-0.44)   | 3.08 ± 0.26 (-0.38)  |
| NLX + VP          | 2 + 100         | 2.98 ± 0.81                | 3.26 ± 0.54 (-0.28)  | 3.20 ± 0.54 (-0.12)   | 3.52 ± 0.71 (-0.54)   | 3.02 ± 0.88 (0.12)    | 2.35 ± 0.72 (-0.73)  |
| NLX + VP          | 2 + 200         | 3.08 ± 0.69                | 2.65 ± 0.69 (0.33)   | 3.23 ± 0.71 (-0.15)   | 3.35 ± 0.66 (-0.37)   | 3.44 ± 0.62 (-0.3)    | 3.15 ± 0.59 (-0.07)  |
| NLX + VP          | 2 + 400         | 2.98 ± 0.74                | 3.22 ± 0.67 (-0.24)  | 3.25 ± 0.64 (-0.17)   | 3.22 ± 0.59 (-0.24)   | 3.48 ± 0.61 (-0.34)   | 3.21 ± 0.33 (-0.13)  |

Abbreviations: NLX, naloxone; VP, *Vernonia patula* ethanol extract.

<sup>a</sup>Values are mean ± SEM (n = 5).

<sup>b</sup>In mL/kg.

\*p < .05 vs control (two-way ANOVA followed by Dunnett's test).

| Treatment         | Dose (mg/kg)    | Time of onset of sleep (min) | Total sleeping time (min) |
|-------------------|-----------------|------------------------------|---------------------------|
| Vehicle (control) | 10 <sup>b</sup> | 14.69 ± 0.94                 | 44.67 ± 2.17              |
| Diazepam          | 1               | 6.34 ± 0.73**                | 96.06 ± 1.39**            |
| VP                | 100             | 10.42 ± 1.21*                | 54.34 ± 2.47*             |
| VP                | 200             | 8.97 ± 1.42*                 | 60.47 ± 2.65**            |
| VP                | 400             | 7.96 ± 1.10**                | 77.40 ± 2.46**            |

Abbreviation: VP, *Vernonia patula* ethanol extract.

<sup>a</sup>Values are mean ± SEM (n = 5).

<sup>b</sup>In mL/kg.

\*p < .05, \*\*p < .01 vs control (one-way ANOVA followed by Dunnett's test).

**TABLE 6** Effect of VP and diazepam in the pentobarbital-induced hypnosis test<sup>a</sup>**TABLE 7** Effects of VP and diazepam on mice in the open field test<sup>a</sup>

| Treatment | Dose (mg/kg)            | Number of movement |               |               |               |               |               |               |
|-----------|-------------------------|--------------------|---------------|---------------|---------------|---------------|---------------|---------------|
|           |                         | 0 min              | 30 min        | 60 min        | 90 min        | 120 min       | 180 min       | 240 min       |
| Vehicle   | 10 <sup>b</sup> (mL/kg) | 116.60 ± 3.08      | 110.68 ± 3.95 | 108.76 ± 3.56 | 106.35 ± 3.58 | 103.39 ± 2.90 | 99.71 ± 2.88  | 96.45 ± 2.59  |
| Diazepam  | 1                       | 91.59 ± 2.35       | 40.13 ± 2.49* | 31.36 ± 2.41* | 26.27 ± 2.29* | 21.11 ± 1.98* | 17.03 ± 1.09* | 7.89 ± 1.20*  |
| VP        | 100                     | 108.30 ± 3.23      | 77.38 ± 2.88* | 67.20 ± 2.79* | 50.65 ± 2.73* | 39.26 ± 2.02* | 28.08 ± 2.43* | 24.27 ± 1.58* |
| VP        | 200                     | 111.30 ± 1.34      | 74.18 ± 2.71* | 65.08 ± 2.77* | 45.85 ± 2.57* | 33.46 ± 1.94* | 27.08 ± 2.47* | 21.07 ± 1.71* |
| VP        | 400                     | 109.19 ± 2.53      | 71.98 ± 2.10* | 59.02 ± 2.32* | 40.06 ± 2.40* | 29.07 ± 1.87* | 25.87 ± 1.72* | 20.19 ± 2.21* |

Abbreviation: VP, *Vernonia patula* ethanol extract.

<sup>a</sup>Values are mean ± SEM (n = 7).

<sup>b</sup>In mL/kg.

\*p < .05 vs control (one-way ANOVA followed by Dunnett's test).

### 3.6 | Pentobarbital-induced hypnosis

At the 400 mg/kg dose, VP significantly decreased the onset of sleep (7.96 min) compared to the control group (14.69 min) and significantly increased the total sleeping time (Table 6).

### 3.7 | Open field test

After 30 min of administration of VP, a noticeable decrease in locomotion in the murine model at all tested doses was observed, which was comparable to the standard diazepam (Table 7).

**TABLE 8** Effects of VP and diazepam on hole cross test<sup>a</sup>

| Treatment | Dose (mg/kg)            | Number of movements |                |                |               |               |               |               |
|-----------|-------------------------|---------------------|----------------|----------------|---------------|---------------|---------------|---------------|
|           |                         | 0 min               | 30 min         | 60 min         | 90 min        | 120 min       | 180 min       | 240 min       |
| Vehicle   | 10 <sup>b</sup> (mL/kg) | 16.55 ± 0.69        | 15.57 ± 0.68   | 14.32 ± 0.65   | 13.34 ± 0.63  | 11.95 ± 0.51  | 9.61 ± 0.73   | 7.74 ± 0.50   |
| Diazepam  | 1                       | 8.92 ± 0.91         | 8.02 ± 0.69**  | 7.19 ± 0.42**  | 6.09 ± 0.34** | 5.47 ± 0.57** | 3.13 ± 0.42** | 2.73 ± 0.24** |
| VP        | 100                     | 14.37 ± 0.27        | 11.53 ± 1.02*  | 10.97 ± 0.74*  | 10.10 ± 0.66* | 9.54 ± 0.62*  | 6.42 ± 0.43*  | 6.01 ± 0.61*  |
| VP        | 200                     | 12.97 ± 0.26        | 11.23 ± 1.08*  | 10.27 ± 0.75** | 9.30 ± 0.70** | 7.80 ± 0.68** | 5.92 ± 0.72*  | 5.23 ± 0.33** |
| VP        | 400                     | 11.89 ± 1.19        | 10.78 ± 0.83** | 9.86 ± 0.73**  | 9.44 ± 0.57** | 7.61 ± 0.49** | 5.46 ± 0.64** | 4.77 ± 0.35** |

Abbreviation: VP, *Vernonia patula* ethanol extract.

<sup>a</sup>Values are mean ± SEM (n = 7).

<sup>b</sup>In mL/kg.

\*p < .05, \*\*p < .01 vs control (one-way ANOVA followed by Dunnett's test).

### 3.8 | Hole cross test

VP at the doses of 200 and 400 mg/kg significantly suppressed both the motor activity and the exploratory behaviour at 90 min and continued up 240 min compared to the standard drug diazepam (Table 8).

### 3.9 | Computational study

Caffeic acid and vanillic acid displayed the highest ligand efficiency indices towards the CB1 receptor (0.0372 and 0.0357, respectively). Both compounds engaged in H-bond and hydrophobic binding interactions with several amino acid residues of CB1 (Table 9; Figures 1 and 2).

### 3.10 | Prediction of the pharmacokinetic properties

All five phenolic compounds showed human intestinal absorption prediction scores similar to dronabinol. Only caffeic acid and vanillic acid showed a positive MLogP, albeit lower than that of THC. Caffeic acid and vanillic acid showed the highest blood-brain barrier (BBB) penetration prediction scores of all phenolics (0.71 and 0.84, respectively) (Table 10).

## 4 | DISCUSSION

VP showed significant antinociceptive activity in the acetic acid-induced writhing test used to evaluate peripheral pain (Gawade, 2012). It showed significant activity in phase 1 and 2 of the formalin-induced pain test (Tjølsen, Berge, Hunskaar, Rosland, & Hole, 1992). In the latter, phase-1 represents central pain (i.e., via the stimulation of sensory afferent C-fibres) whilst phase-2 represents inflammatory pain (i.e., via the production of prostaglandins and bradykinins in peripheral tissues) (Chen, Tsai, & Wu, 1995). VP also

showed significant antinociceptive activity in the hot plate test (only at 200 and 400 mg/kg) and the tail immersion tests. These tests evaluate central analgesic activity linked to supra-spinal and spinal reflex, respectively, through the modulation of  $\mu$  opioid (MOP) receptors (Jinsmaa et al., 2005). Co-administration of the opioid receptor antagonist naloxone, which reverses the action of morphine on MOP receptors, led to a reversal of the analgesic effect of VP. Interestingly, it has been suggested that the MOP and CB1 receptors mediate some overlapping pharmacological responses, including pain (Rios, Gomes, & Devi, 2006). The traditional use of VP to treat convulsions, and the fact that other species of *Vernonia* have displayed sedative activity, prompted us to further investigate the neuropharmacological effects of VP. In the pentobarbital-induced hypnosis test, VP showed a significant and dose-dependent sedative effect. Its CNS-depressant activity was demonstrated by a decrease in locomotor activity and exploratory behaviour in mice in the open field and hole cross tests, respectively (Howlader et al., 2017).

HPLC-DAD analysis indicated the presence of caffeic acid, gallic acid, vanillic acid, quercetin and kaempferol in the aerial parts of VP. Caffeic acid has previously been reported for its antinociceptive properties in vivo (Mehrotra, Shanbhag, Chamallamudi, Singh, & Mudgal, 2011; Park et al., 2011). Quercetin has also been reported to induce analgesia in several models of pain (Filho, Filho, Olinger, & de Souza, 2008). Our results are in agreement with studies on other medicinal plants containing phenolic compounds (including vanillic acid, gallic acid, caffeic acid, quercetin and kaempferol) that displayed significant antinociceptive and sedative activity in vivo (Aguirre-Hernández et al., 2010; Anzoise, Marrassini, Ferraro, & Gorzalczany, 2013; Khatun et al., 2016; Rahman, Khatun, Nesa, Hosain, & Jahan, 2015).

Cannabinoid agonists (e.g., THC) have demonstrated great potential for the treatment of pain, inflammation and neurological disorders (Cheng & Hitchcock, 2007; Koob, 2006; Pacher, Bátkai, & Kunos, 2006; Pertwee, 2006; Savage, 2009; Williams et al., 2013). Their interaction with the cannabinoid receptors leads to a decrease in intracellular cAMP levels. Low concentrations of cAMP leads to a decrease in presynaptic entry of calcium ions, which results in a reduction in the release of neurotransmitters such as GABA, L-glutamate,

**TABLE 9** Docking scores, ligand efficiency (LE<sup>a</sup>) indices and molecular interactions of VP phenolics towards the CB1 receptor target

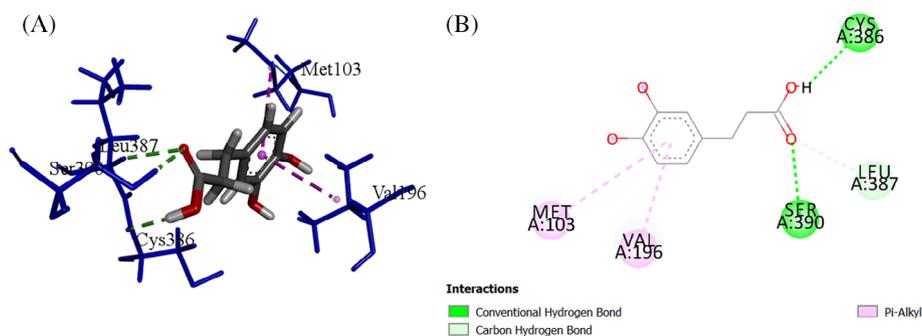
| Ligand        | Docking score ( $\Delta G$ in kcal/mol) | LE index <sup>a</sup> | Interacting residues | Distance (Å) | Category      | Type                   |
|---------------|---|-----------------------|----------------------|--------------|---------------|------------------------|
| Caffeic acid  | −6.7                                    | <b>0.0372</b>         | CYS386               | 2.171        | H-bond        | Conventional           |
|               |   |                       | SER390               | 2.201        | H-bond        | Conventional           |
|               |   |                       | LEU387               | 2.704        | H-bond        | C–H bond               |
|               |   |                       | VAL196               | 4.095        | Hydrophobic   | Pi-alkyl               |
|               |   |                       | MET103               | 5.391        | Hydrophobic   | Pi-alkyl               |
| Vanillic acid | −6.0                                    | <b>0.0357</b>         | ASN101               | 2.333        | H-bond        | Conventional           |
|               |   |                       | SER383               | 2.504        | H-bond        | C–H bond               |
|               |   |                       | PRO269               | 2.987        | H-bond        | C–H bond               |
|               |   |                       | PHE108               | 5.238        | Hydrophobic   | Pi-pi T-shaped         |
|               |   |                       | PHE268               | 5.432        | Hydrophobic   | Pi-pi T-shaped         |
| Gallic acid   | −5.6                                    | <b>0.0329</b>         | PHE102               | 5.650        | Hydrophobic   | Pi-pi T-shaped         |
|               |   |                       | ASP104               | 1.962        | H-bond        | Conventional           |
|               |   |                       | ASP104               | 2.167        | H-bond        | Conventional           |
|               |   |                       | SER383               | 2.645        | H-bond        | C–H bond               |
| Quercetin     | −7.5                                    | <b>0.0248</b>         | PHE102               | 5.793        | Hydrophobic   | Pi-pi T-shaped         |
|               |   |                       | ASP403               | 2.120        | H-bond        | Conventional           |
|               |   |                       | ARG214               | 2.710        | H-bond        | Conventional           |
|               |   |                       | SER152               | 2.910        | H-bond        | Pi-donor hydrogen bond |
|               |   |                       | ASP338               | 3.730        | Electrostatic | Pi-anion               |
|               |   |                       | ARG150               | 4.090        | H-bond        | Pi-cation              |
|               |   |                       | ARG340               | 4.120        | Hydrophobic   | Pi-alkyl               |
|               |   |                       | ARG150               | 4.250        | Electrostatic | Pi-cation              |
|               |   |                       | LEU341               | 4.780        | Hydrophobic   | Pi-alkyl               |
|               |   |                       | MET337               | 4.880        | Hydrophobic   | Pi-alkyl               |
|               |   |                       | LEU341               | 5.140        | Hydrophobic   | Pi-alkyl               |
|               |   |                       | MET337               | 5.370        | Hydrophobic   | Pi-alkyl               |
|               |   |                       | TYR153               | 5.400        | Hydrophobic   | Pi-pi T-shaped         |
| LEU341        | 5.440                                   | Hydrophobic           | Pi-alkyl             |              |               |                        |
| TYR153        | 5.840                                   | Hydrophobic           | Pi-pi T-shaped       |              |               |                        |
| Kaempferol    | −8.2                                    | <b>0.0286</b>         | ASP104               | 2.186        | H-bond        | Conventional           |
|               |   |                       | VAL196               | 4.671        | Hydrophobic   | Pi-alkyl               |
|               |   |                       | PHE268               | 4.852        | Hydrophobic   | Pi-pi T-shaped         |
|               |   |                       | MET103               | 4.938        | Hydrophobic   | Pi-alkyl               |
|               |   |                       | MET103               | 4.984        | Hydrophobic   | Pi-alkyl               |
|               |   |                       | CYS386               | 5.483        | Hydrophobic   | Pi-alkyl               |
|               |   |                       | PHE108               | 5.800        | Hydrophobic   | Pi-pi T-shaped         |

<sup>a</sup>LE =  $-\Delta G$ /molecular weight of the ligand. Dronabinol (THC) had a docking score of  $-7.5$  kcal/mol and a LE index of 0.0239.

noradrenaline, dopamine, serotonin and acetylcholine (Howlett et al., 2002; Lu & Mackie, 2016). A molecular docking of VP phenolics towards the CB1 receptor was performed in order to assess the role of the cannabinoid system in the observed pharmacological effects of VP. Caffeic acid and vanillic acid showed the strongest CB1-ligand efficiency indices.

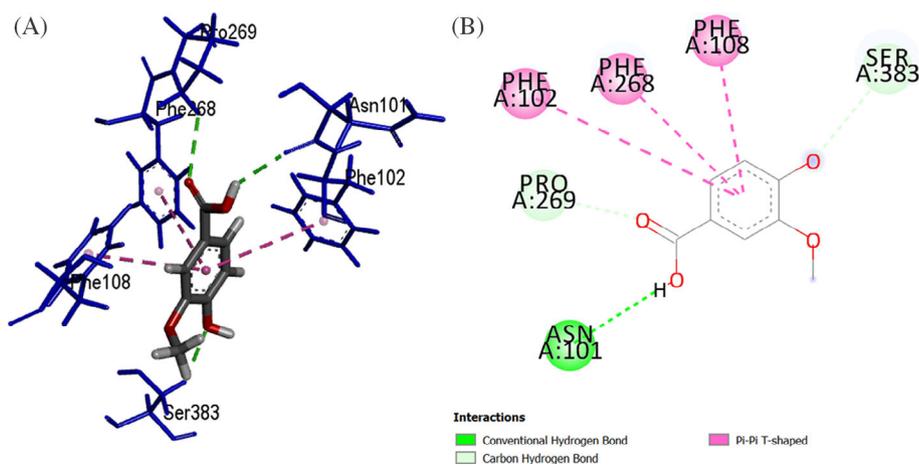
In ADMET studies, the MLogP was used to predict the ability of each phenolic to penetrate the BBB (Sterling et al., 2014). Only caffeic

acid and vanillic acid showed a positive MLogP prediction score amongst the phenolic compounds tested. Vanillic acid also showed the lowest topological polar surface area and a good predicted ability to penetrate the BBB, which is critical for any compound with central analgesic activity. Interestingly, recent work has demonstrated that protocatechuic acid, an analogue of vanillic acid, induced analgesia in vivo via activation of the CB1 receptors (Dikmen, Okcay, Arslan, & Bektas, 2019).



**FIGURE 1** (A) Docked pose of caffeic acid in the CB1 receptor binding site, showing molecular interactions (hydrogen/hydrophobic bonds as green and pink dashed lines, respectively); (B) 2D plot of interactions between caffeic acid and key residues of the CB1 receptor generated by BIOVIA Discovery Studio visualizer. The solvent accessible surface is depicted as a background grey circle with the radius proportional to the exposure [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

**FIGURE 2** (A) Docked pose of vanillic acid in the CB1 receptor binding site, showing molecular interactions (hydrogen/hydrophobic bonds as green and pink dashed lines, respectively); (B) 2D plot of interactions between vanillic acid and key residues of the CB1 receptor generated by BIOVIA Discovery Studio visualizer. The solvent accessible surface is depicted as a background grey circle with the radius proportional to the exposure [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**TABLE 10** Drug-likeness parameters prediction for dronabinol and VP phenolics

| Parameters                         | Dronabinol (THC)                               | Gallic acid                                  | Vanillic acid                                | Caffeic acid                                 | Quercetin                                      | Kaempferol                                     |
|------------------------------------|--|--|--|--|--|--|
| Structural formula                 | C <sub>21</sub> H <sub>30</sub> O <sub>2</sub> | C <sub>7</sub> H <sub>6</sub> O <sub>5</sub> | C <sub>8</sub> H <sub>8</sub> O <sub>4</sub> | C <sub>9</sub> H <sub>8</sub> O <sub>4</sub> | C <sub>15</sub> H <sub>10</sub> O <sub>7</sub> | C <sub>15</sub> H <sub>10</sub> O <sub>6</sub> |
| Molecular weight (g/mol)           | 314.46   | 170.12                                       | 168.15                                       | 180.16                                       | 302.23   | 286.24   |
| BBB permeability (p.s.)            | 0.99   | 0.71   | 0.84   | 0.44   | 0.46   | 0.56   |
| MLogP                              | 4.39   | NSP  | 0.74   | 0.7  | NSP  | NSP  |
| TPSA (Å <sup>2</sup> )             | 29.46  | 97.99  | 66.76  | 77.76  | 131.36   | 111.13   |
| Human intestinal absorption (p.s.) | 0.99   | 0.98   | 0.99   | 0.96   | 0.98   | 0.99   |
| Human oral bioavailability         | NSP  | 0.7143                                       | 0.7429                                       | 0.6429                                       | 0.5429   | 0.6286   |
| Bioavailability score              | 0.58   | 0.56   | 0.56   | 0.56   | 0.55   | 0.55   |

Abbreviations: BBB, blood–brain barrier, MLogP, LogP<sub>oil/water</sub>; NSP, negative score prediction; p.s., prediction score; TPSA, topological polar surface area.

## 5 | CONCLUSION

Caffeic acid, gallic acid, vanillic acid, quercetin and kaempferol were detected in the ethanol extract of the aerial parts of VP. This extract demonstrated significant antinociceptive and CNS-depressant activity in mice. A molecular docking of these phenolics towards the CB1cannabinoid receptor revealed that caffeic acid and vanillic acid had the strongest CB1-ligand efficiency indices. These findings justify

the traditional use of VP to treat pain. They suggest a role for its phenolic compounds in mediating analgesia, including via the cannabinoidergic system, which warrants further in vivo investigations.

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## CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

## AUTHOR CONTRIBUTIONS

MAS designed and performed the computational studies. MSIH conducted the in-vivo studies. MSR and VS analysed the data, organised the references and performed the statistical calculations. MAS and VS wrote the manuscript. JAS supervised the project. All the authors reviewed the content of the manuscript.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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