RESEARCH PAPER

Effectiveness of Cocoa Extract as an Adjunctive Therapy to Pregabalin on Monocyte Chemoattractant Protein-1 (MCP-1) Levels and Pain Scores in an Animal Model of Neuropathic Pain

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ABSTRACT

Background: Neuropathic pain is a chronic pain condition that is difficult to manage and is often associated with neuroinflammatory processes, including increased levels of Monocyte Chemotactic Protein-1 (MCP-1). Pregabalin is a first-line therapy for neuropathic pain; however, its effectiveness is limited by dose-related adverse effects. Cocoa contains bioactive compounds with anti-inflammatory and antinociceptive properties, suggesting its potential use as an adjuvant therapy.

Method: This experimental study employed a randomized post-test only design using a mouse model of neuropathic pain induced by chronic constriction injury (CCI). Animals were divided into several treatment groups: K0 as the negative control group without treatment; K1 receiving pregabalin at a dose of 60 mg/kgBW; K2 receiving a combination of pregabalin 60 mg/kgBW and cocoa extract at a dose of 1 mg/gBW; and K3 receiving a half-dose of pregabalin (30 mg/kgBW) combined with cocoa extract at a dose of 1 mg/gBW. Pain assessment was performed using the von Frey test, while serum MCP-1 levels were measured using enzyme-linked immunosorbent assay (ELISA).

Results: Administration of cocoa extract as an adjuvant to pregabalin 60mg/kgBW resulted in higher mechanical pain thresholds and greater reductions in MCP-1 levels compared to groups without adjuvant therapy.

Conclusion: Cocoa extract has the potential to enhance the effectiveness of pregabalin in reducing neuropathic pain through modulation of MCP-1 in a CCI animal model....

Keywords: neuropathic pain; cocoa extract; pregabalin; MCP-1; chronic constriction injury

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INTRODUCTION

Neuropathic pain is a chronic pain condition arising from a lesion or disease affecting the somatosensory nervous system. Globally, its prevalence is estimated to range from 6% to 8% among the adult population, with variations influenced by demographic factors and underlying clinical conditions [1]. In Indonesia, a multicenter study involving 13 hospitals reported that 21.8% of patients presenting with pain were diagnosed with neuropathic pain, highlighting its substantial clinical burden within national healthcare settings [2]. Beyond pain itself, neuropathic pain is frequently accompanied by sleep disturbances, anxiety, and depression, leading to significant impairment in quality of life and increased healthcare utilization, including higher rates of analgesic prescriptions [3].

Pharmacological therapy remains the cornerstone of neuropathic pain management; however, the effectiveness of monotherapy is limited, with reported response rates of only approximately 25–40%. This limited efficacy is largely attributed to dose constraints imposed by adverse effects, particularly during long-term treatment [4]. Consequently, combination therapy strategies have gained increasing attention, aiming to achieve superior analgesic effects through synergistic mechanisms while enabling dose reduction and improved tolerability [5].

From a pathophysiological perspective, neuropathic pain involves complex interactions between neuronal hyperexcitability and neuroinflammatory processes. A key mediator in this cascade is monocyte chemotactic protein-1 (MCP-1), also known as CCL2. MCP-1 is a chemokine that plays a crucial role in recruiting and activating monocytes and other immune cells at sites of nerve injury, both in the peripheral and central nervous systems. Increased MCP-1 expression has been consistently demonstrated in the dorsal root ganglion and dorsal horn of the spinal cord in various neuropathic pain models and has been shown to correlate

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with mechanical allodynia and hyperalgesia [6,7]. Furthermore, activation of the MCP-1/CCR2 signaling pathway enhances nociceptive neuronal excitability and central sensitization, thereby contributing to the persistence of chronic pain [8,9]. These findings suggest that MCP-1 is not only a key pathophysiological mediator but also a potential biological marker for assessing neuroinflammatory activity and therapeutic response in neuropathic pain.

In current clinical practice, gabapentinoids are recommended as first-line agents for neuropathic pain by the Special Interest Group on Neuropathic Pain (NeuPSIG). Pregabalin and gabapentin, both approved by the Food and Drug Administration (FDA), exert their analgesic effects by binding to the α2δ subunit of voltage-gated calcium channels, thereby reducing presynaptic calcium influx and inhibiting the release of excitatory neurotransmitters [10,11]. Compared with gabapentin, pregabalin exhibits a higher binding affinity to this subunit, resulting in more consistent analgesic efficacy [12]. Nevertheless, the clinical use of pregabalin remains limited by dose-related adverse effects, particularly at higher doses and with prolonged administration, underscoring the need for effective adjuvant therapies.

Alongside limitations of conventional pharmacological treatments, natural compounds such as cocoa have emerged as potential adjuvant agents in pain management. Cocoa contains a wide range of phytochemicals, particularly polyphenols, with documented anti-inflammatory, antioxidant, and pain-modulating properties. Additional components, including methylxanthines, flavan-3-ols, stilbenoids, phenolic acid derivatives, amines, and alkaloids, have been shown to influence pain perception through multiple biological pathways [13,14]. Experimental evidence indicates that cocoa-rich diets can attenuate inflammatory responses and neurogenic pain behaviors in animal models, supporting its potential role as an adjunctive therapy in neuropathic pain management [15,16].

To experimentally evaluate pain mechanisms and therapeutic efficacy, animal models play a critical role. The chronic constriction injury (CCI) model is one of the most widely used peripheral neuropathic pain models, producing partial nerve injury that triggers inflammatory responses, glial activation, and neuronal hyperexcitability without complete nerve transection. Behaviorally, CCI induces mechanical allodynia and hyperalgesia within 3–8 days after injury, with persistence into subacute and chronic phases, closely resembling clinical features of human neuropathic pain [9,17]. Importantly, CCI has been shown to upregulate neuroinflammatory mediators, including MCP-1, in the dorsal root ganglion and spinal cord, making it a suitable model for investigating the relationship between inflammatory biomarkers and pain behavior [18].

Based on this background, the present study aimed to evaluate the effectiveness of cocoa extract as an adjunct to pregabalin on mechanical pain sensitivity and serum MCP-1 levels in a mouse model of neuropathic pain induced by chronic constriction injury. The findings are expected to provide insights into the role of natural compound-based combination therapy in modulating neuroinflammation and neuropathic pain, and to support the development of safer and more effective therapeutic strategies.

METHOD

Study Design

This study was a true experimental laboratory-based study employing a randomized post-test only control group design. The post-test only approach was selected to minimize repeated handling and stress-related physiological alterations in experimental animals, which could confound inflammatory and nociceptive outcomes. The experimental workflow consisted of baseline behavioral assessment, induction of neuropathic pain, confirmation of pain development, random allocation to intervention groups, post-treatment behavioral evaluation, and biomarker analysis.

Experimental Animals and Housing Conditions

Male white mice (Mus musculus), aged 8–10 weeks and weighing 25–35 g, were used as experimental subjects. Animals were obtained from the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia. Prior to experimentation, mice were acclimatized for 7 days in standard laboratory cages under controlled environmental conditions. Animals were maintained on a 12-hour light/dark cycle (light phase from 06:00 to 18:00) with ad libitum access to standard feed and drinking water. Only healthy animals, defined by stable body weight, clear eyes, and normal fur condition, were included in the study.

Inclusion and Exclusion Criteria

Inclusion criteria comprised male Mus musculus with body weight between 25 and 35 g and good general health. Animals were excluded from the study if they died during the experimental period, developed illness, or failed to exhibit neuropathic pain behavior following chronic constriction injury induction.

Sample Size Determination

Sample size calculation was based on a hypothesis testing formula for comparison of mean values across multiple groups, with a significance level (α) of 0.05 and statistical power (1−β) of 0.80. With four experimental groups and an estimated effect size based on Cohen's f, the minimum required sample size was calculated as six animals per group. To account for potential attrition, the sample size was increased to seven animals per group, resulting in a total of 28 mice.

Randomization and Group Allocation

After confirmation of neuropathic pain development, animals were randomly allocated into four experimental groups (n = 7 per group) using simple randomization. The groups were defined as follows: a placebo control group receiving vehicle only, a pregabalin monotherapy group receiving 60 mg/kg body weight, a combination therapy group receiving pregabalin 60 mg/kg plus cocoa extract 1

mg/g body weight, and a second combination therapy group receiving pregabalin 30 mg/kg plus cocoa extract 1 mg/g body weight.

Induction of Neuropathic Pain

Neuropathic pain was induced using the chronic constriction injury (CCI) model of the right sciatic nerve. All surgical procedures were performed by a single veterinarian with more than five years of experience to reduce inter-operator variability. General anesthesia was administered via intraperitoneal injection of a ketamine–xylazine–acepromazine cocktail at doses of 90 mg/kg, 10 mg/kg, and 1 mg/kg, respectively. After a skin incision at the mid-thigh level, the sciatic nerve was exposed and carefully freed from surrounding tissue proximal to its trifurcation. Three loose ligatures were placed around the nerve using 6-0 silk monofilament sutures, with approximately 1 mm spacing between ligatures. The incision was closed with sutures and sterile dressing. Neuropathic pain development was confirmed seven days postoperatively using mechanical pain assessment.

Preparation of Study Compounds

Pregabalin tablets were crushed into fine powder, and 100 mg of active substance was weighed and suspended in distilled water containing 1% sodium carboxymethyl cellulose (CMC-Na) to obtain a homogeneous suspension. Cocoa extract was prepared from roasted cocoa beans sourced from the Indonesian Coffee and Cocoa Research Institute (Puslitkoka), Jember, Indonesia. The beans were ground into powder and extracted using 70% ethanol. The resulting extract was then suspended in 1% CMC-Na to achieve a final concentration of 100 mg/mL and stored in sterile vials until use.

Treatment Administration

Following confirmation of neuropathic pain, animals received treatments according to their assigned groups. Treatments were administered orally using a feeding needle twice daily for seven consecutive days. The control group received vehicle only, whereas treatment groups received pregabalin alone or in combination with cocoa extract at predetermined doses. Treatment duration and dosing regimens were consistent across all groups.

Assessment of Mechanical Pain Sensitivity

Mechanical pain sensitivity was evaluated using an electronic von Frey apparatus. Mice were placed individually on an elevated wire mesh platform and allowed to acclimatize for one hour prior to testing. A von Frey filament was applied perpendicularly to the plantar surface of the hind paw with gradually increasing force until a withdrawal response was elicited. The applied force at withdrawal was automatically recorded. Measurements were obtained at baseline, seven days after CCI to confirm neuropathic pain induction, and after completion of the treatment period. Higher withdrawal thresholds indicated lower pain sensitivity.

Measurement of Serum MCP-1 Levels

Blood samples were collected at the end of the treatment period. Animals were anesthetized and humanely euthanized prior to blood collection. Samples were centrifuged at 1,000 × g for 30 minutes at 4°C to obtain serum. Serum concentrations of monocyte chemotactic protein-1 (MCP-1) were determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit in accordance with the manufacturer's instructions. Absorbance was measured at a wavelength of 450 nm using a microplate reader, and MCP-1 concentrations were expressed in ng/mL.

Data Collection and Statistical Analysis

All experimental data were recorded using standardized data collection forms. Data were processed using Microsoft Excel and analyzed with SPSS Statistics software. Normality of data distribution was assessed using the Shapiro–Wilk test, and homogeneity of variance was evaluated using Levene's test. Comparisons among groups were performed using one-way analysis of variance (ANOVA) for normally distributed data or the Kruskal–Wallis test for non-normally distributed data. Within-group comparisons were analyzed using paired t-tests or Wilcoxon signed-rank tests as appropriate. Correlations between mechanical pain thresholds and serum MCP-1 levels were analyzed using Pearson or Spearman correlation tests depending on data distribution. A p-value of less than 0.05 was considered statistically significant.

Ethical Approval

All experimental procedures involving animals were conducted in accordance with institutional and national guidelines for the care and use of laboratory animals and complied with internationally accepted principles of humane animal research. Ethical approval for this study was granted by the Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia (Ethical Clearance No.: 2.KEH.78.05.2025).

RESULTS

A total of 28 male mice completed the study and were included in the final analysis. All animals met the inclusion criteria and successfully developed unilateral neuropathic pain following CCI. Baseline characteristics and pre-intervention mechanical pain thresholds were comparable across all experimental groups. As summarized in Table 1, no statistically significant differences were observed in baseline von Frey withdrawal thresholds between groups for either the right or left hind paw, confirming homogeneity of mechanical sensitivity prior to CCI induction.

Table 1. Baseline characteristics and baseline/post-CCI von Frey withdrawal thresholds

Variable	K0 (Placebo)	K1 (Pregabalin 60 mg/kg)	K2 (Pregabalin 60 mg/kg + Cocoa)	K3 (Pregabalin 30 mg/kg + Cocoa)	*p*-value
Number of animals (n)	7	7	7	7	—
Age (weeks)	8–12	8–12	8–12	8–12	—
Body weight (g)	25–30	25–30	25–30	25–30	—
Baseline von Frey – Right paw (gf)	4.48 ± 0.46	4.58 ± 0.47	4.50 ± 0.41	4.67 ± 0.43	0.864 †
Baseline von Frey – Left paw (gf)	4.33 ± 0.41	4.47 ± 0.45	4.66 ± 0.44	4.53 ± 0.38	0.563 †
Post-CCI von Frey – Right paw (gf)	1.22 ± 0.25	1.22 ± 0.24	1.12 ± 0.23	1.22 ± 0.21	1.000 †
Post-CCI von Frey – Left paw (gf)	4.46 ± 0.47	4.54 ± 0.46	4.33 ± 0.40	4.34 ± 0.46	0.810 †

Values are presented as mean ± SD.
†One-way ANOVA. No significant between-group differences were observed at baseline or post-CCI.

Following CCI, all animals demonstrated a marked reduction in mechanical withdrawal thresholds in the ipsilateral (right) hind paw, confirming successful induction of neuropathic pain. Importantly, there were no between-group differences at the post-CCI time point, indicating that the severity of neuropathic pain was comparable before treatment initiation (data shown in Table 1).

After seven days of intervention, significant differences in mechanical pain thresholds were observed among treatment groups in the right hind paw. As presented in Table 2, the placebo group exhibited persistently low withdrawal thresholds, whereas all active treatment groups demonstrated significantly higher thresholds, indicating attenuation of mechanical allodynia. Post-hoc analysis revealed that pregabalin monotherapy significantly improved pain thresholds compared with placebo, while both combination regimens (pregabalin plus cocoa extract) produced greater analgesic effects than pregabalin alone. Furthermore, the combination of cocoa extract with full-dose pregabalin resulted in a significantly higher withdrawal threshold than the half-dose pregabalin combination, suggesting a dose-dependent synergistic effect. In contrast, no significant differences were detected among groups in the contralateral (left) hind paw, consistent with the unilateral nature of the CCI model.

Within-group comparisons demonstrated that active treatments significantly increased mechanical withdrawal thresholds compared with the post-CCI state, whereas no significant change was observed in the placebo group, further supporting the analgesic efficacy of pregabalin and its enhancement by cocoa extract (Table 2).

Table 2. Post-treatment von Frey withdrawal thresholds and post-hoc comparisons (right paw)

A. Overall comparison

Group	Von Frey threshold (gf)	Statistical test	*p*-value
K0	1.04 (0.85–1.33)	Kruskal–Wallis	<0.001
K1	2.57 (1.95–2.96)		
K2	3.55 (3.39–4.09)		
K3	3.28 (3.12–3.79)		

Values are presented as median (min–max).

B. Post-hoc pairwise comparisons (Mann–Whitney test)

Comparison	*p*-value
K0 vs K1	0.002
K0 vs K2	0.002
K0 vs K3	0.002
K1 vs K2	0.002
K1 vs K3	0.002
K2 vs K3	0.018

Serum MCP-1 concentrations differed significantly across groups following treatment. As shown in Table 3, one-way ANOVA demonstrated a significant overall group effect, with the highest MCP-1 levels observed in the placebo group and progressively lower levels in the pregabalin and combination therapy groups. Post-hoc comparisons indicated that all treatment groups had significantly lower MCP-1 concentrations than placebo, with the greatest reduction observed in the group receiving cocoa extract combined with full-dose pregabalin. These findings suggest that the addition of cocoa extract enhanced the anti-

inflammatory effect of pregabalin, particularly at the full therapeutic dose.

Table 3. Serum MCP-1 levels and post-hoc analysis

A. Group comparison

Group	MCP-1 (pg/mL)	*p*-value (ANOVA)
K0	98.56 ± 7.98	<0.001
K1	76.16 ± 7.38	
K2	57.36 ± 6.28	
K3	84.55 ± 4.23	
Values are presented as mean ± SD.		

B. Post-hoc analysis (LSD test)

Comparison	Mean difference	95% CI	*p*-value
K0 vs K1	22.40	15.08 – 29.71	<0.001
K0 vs K2	41.20	33.88 – 48.51	<0.001
K0 vs K3	14.01	6.69 – 21.32	<0.001
K1 vs K2	18.80	11.48 – 26.11	<0.001
K1 vs K3	−8.39	−15.70 – −1.07	0.030
K2 vs K3	−27.19	−34.50 – −19.87	<0.001

Correlation analysis demonstrated a strong inverse relationship between serum MCP-1 concentrations and post-treatment mechanical pain thresholds. As summarized in Table 4, higher MCP-1 levels were significantly associated with lower von Frey thresholds, indicating greater pain sensitivity, whereas lower MCP-1 levels correlated with improved mechanical tolerance.

Table 4. Correlation between serum MCP-1 levels and post-treatment von Frey thresholds

Variables	*n*	Correlation coefficient (r)	*p*-value
MCP-1 vs von Frey (right paw)	24	−0.710	<0.001
Pearson correlation analysis.			

DISCUSSION

This randomized post-test–only experimental study in a murine CCI model demonstrates that adding cocoa extract to pregabalin meaningfully improves neuropathic pain–related outcomes at both behavioural and biological levels. After confirming successful and homogeneous induction of ipsilateral mechanical allodynia by CCI, we observed that post-treatment mechanical withdrawal thresholds (right paw, primary outcome) were significantly higher in all active-treatment groups than placebo, with the greatest improvement in the pregabalin 60 mg/kg plus cocoa extract group. In parallel, serum MCP-1 (CCL2), a key neuroinflammatory chemokine implicated in neuropathic pain maintenance, differed significantly among groups, with the lowest levels again observed in the pregabalin 60 mg/kg plus cocoa extract group. Importantly, MCP-1 levels correlated strongly and inversely with post-treatment von Frey thresholds, supporting the study hypothesis that adjunct cocoa extract enhances analgesic response in part through modulation of MCP-1–related neuroinflammation [6–9].

The validity of the pain model and behavioural outcome measure is supported by the clear ipsilateral reduction in mechanical thresholds after CCI without contralateral changes, a pattern consistent with the established CCI phenotype of mechanical allodynia driven by peripheral nerve injury, local immune activation, and central sensitisation [19–21]. von Frey testing remains a widely accepted method for quantifying mechanical allodynia in rodent neuropathic pain, although its performance can be influenced by environmental conditions and operator technique; therefore, standardisation and experienced assessors are essential to reduce measurement variability [22,23]. The absence of between-group differences in baseline and post-CCI thresholds before treatment indicates that randomisation produced comparable groups and that the treatment effects observed after intervention are unlikely to be explained by baseline imbalance.

Regarding treatment effects, pregabalin monotherapy increased right-paw mechanical thresholds compared with placebo, consistent with guideline-supported clinical efficacy of gabapentinoids in neuropathic pain and their established mechanism of binding the α2-δ subunit of voltage-gated calcium channels, thereby reducing excitatory neurotransmitter release and nociceptive transmission [10,12,24]. Notably, both combination regimens (cocoa extract plus pregabalin) outperformed pregabalin alone in post-hoc comparisons, suggesting an additive or synergistic interaction. The superiority of the full-dose pregabalin combination over the half-dose pregabalin combination implies a dose–response component where cocoa extract augments pregabalin's analgesic effect within the 7-day treatment window. Nevertheless, the finding that cocoa extract combined with half-dose pregabalin still achieved higher thresholds than pregabalin alone is clinically relevant, because dose-limiting adverse effects are a major barrier to long-term gabapentinoid therapy and combination strategies are often explored to maintain efficacy while improving tolerability [4,5].

Biologically, MCP-1 is a plausible mechanistic bridge between peripheral nerve injury and sustained pain hypersensitivity. After nerve injury, MCP-1/CCR2 signalling contributes to immune cell recruitment, glial activation, and heightened excitability within nociceptive circuits, thereby reinforcing central sensitisation and persistent allodynia [6–9]. In this study, serum MCP-1 differed significantly among groups with clear pairwise separation, and the combination of cocoa extract with pregabalin 60 mg/kg produced the greatest reduction. The strong negative correlation between MCP-1 and von Frey thresholds aligns with prior observations that MCP-1 tracks neuroinflammatory activity and pain-like behaviour in neuropathic pain models, supporting its potential utility as a translational biomarker of treatment response. While

serum MCP-1 does not localise the inflammatory source (peripheral nerve vs dorsal root ganglion vs spinal cord), the directionality and strength of association observed here provide convergent evidence that treatments which suppress MCP-1–linked inflammatory signalling are accompanied by functional improvements in mechanical thresholds [6,7].

The adjunct effect of cocoa extract is biologically credible given its complex phytochemical profile. Cocoa contains abundant polyphenols and other bioactive compounds with antioxidant and anti-inflammatory properties that may attenuate neuroinflammation, reduce oxidative stress, and modulate nociceptive signalling pathways [13]. Preclinical work has reported that cocoa-rich interventions can dampen inflammatory responses and neurogenic pain behaviours, lending support to cocoa-derived products as potential adjuvants in pain management [15]. Within the present framework, cocoa extract may reduce MCP-1 production directly (e.g., via suppression of pro-inflammatory transcriptional activity) or indirectly by limiting upstream cytokine signalling and glial activation, thereby complementing pregabalin's synaptic modulation. The more pronounced MCP-1 suppression in the full-dose pregabalin plus cocoa group compared with other regimens suggests that simultaneous dampening of neuronal hyperexcitability (pregabalin) and inflammatory/oxidative pathways (cocoa) may be necessary to achieve maximal short-term neuroinflammatory downregulation. However, the non-linear relationship between molecular mediators and behavioural outcomes should be acknowledged: neuropathic pain is multifactorial, and functional recovery likely reflects integrated effects across neuronal, immune, and glial pathways rather than a single mediator alone [1,3].

This study has several strengths. The experimental design used a well-characterised CCI model and included a placebo control and active comparator (pregabalin), enabling clear attribution of incremental benefit to the cocoa extract adjunct. Baseline and post-CCI equivalence across groups supports internal validity, and the study paired behavioural outcomes with a mechanistically relevant biomarker (MCP-1), strengthening biological interpretation. Nonetheless, limitations should temper interpretation. First, the study used only male mice of a single strain and age range; sex- and strain-dependent differences in pain processing and neuroimmune responses may limit generalisability. Second, CCI ligation tension was standardised procedurally but not quantified mechanically, which could introduce variability in injury severity despite experienced operators. Third, pain assessment focused on mechanical allodynia; thermal hyperalgesia and spontaneous pain–related behaviours were not measured, so the findings primarily reflect one sensory modality [23]. Fourth, the post-test–only design precluded within-animal longitudinal MCP-1 profiling, limiting causal inference about temporal coupling between biomarker change and behavioural recovery. Finally, serum MCP-1 provides systemic readout and may not perfectly mirror local neuroinflammatory changes within dorsal root ganglia or spinal cord.

This study has several limitations. It was conducted in a single animal species and sex using a murine CCI model, which may limit generalisability to other species and to clinical neuropathic pain. The tension of sciatic nerve ligation was not mechanically quantified, potentially introducing variability in injury severity. Pain assessment was limited to mechanical allodynia using the von Frey test, and longitudinal measurement of MCP-1 was not performed. Future studies should include both sexes, standardised injury induction, multimodal pain assessments, and longitudinal as well as tissue-level biomarker analyses to further clarify mechanisms and translational relevance of cocoa-based adjuvant therapy.

CONCLUSIONS

Adjunctive cocoa extract enhances the analgesic effect of pregabalin in a murine chronic constriction injury model of neuropathic pain, as reflected by improved mechanical pain thresholds and greater reductions in serum MCP-1 compared with pregabalin monotherapy. These findings indicate that cocoa extract has the potential to enhance the effectiveness of pregabalin in reducing neuropathic pain through modulation of MCP-1–related neuroinflammatory pathways. Further mechanistic and translational studies are needed to clarify its clinical relevance and potential role in optimising neuropathic pain therapy.

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