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Serotonin release mediates analgesia via opioidergic system and withdrawal symptoms in chronic kratom extract-treated mice

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Abstract

Background Kratom alleviates pain by activating μu-opioid receptors (MOR), which trigger serotonin release to produce analgesia. Serotonin also interferes drug abuse effect. This study aimed to determine the role of serotonin in kratom-induced pain relief and withdrawal symptoms in mice.

Methods The analgesic effect was assessed using the hot-plate test. To induce withdrawal symptoms, mice received naloxone after being treated with kratom extracts for five days at increasing doses. Another group of morphine-dependent mice was treated with kratom extracts to ameliorate their withdrawal symptoms. A molecular docking study and molecular dynamics were conducted to predict the binding target of alkaloid kratom for increasing serotonin levels.

Results Chronic administration of kratom alkaloid extract (20 mg/kg) produced analgesic effects comparable to morphine (10 mg/kg). In contrast, kratom crude extracts (10 mg/kg and 20 mg/kg) demonstrated lower analgesia activity. This analgesic effect was mediated by MOR activation, leading to decreased intracellular cAMP and increased serotonin transmission. Repeated and increasing doses of crude or alkaloid kratom extracts (8 mg/kg to 45 mg/kg) produced less severe withdrawal symptoms than morphine. Increased dopamine and serotonin levels contributed to the onset of withdrawal symptoms. In the morphine group, treatment with kratom extracts increased serotonin levels while reducing dopamine. Molecular docking and molecular dynamics result revealed that kratom alkaloids interacts more readily with tryptophan hydroxylase, the enzyme responsible for serotonin biosynthesis.

Conclusions Kratom extracts have the potential to provide analgesic effects and withdrawal symptoms, both of which are mediated by elevated serotonin release.

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Highlights

- The alkaloid extract of kratom produces an analgesic effect similar to morphine but with a longer duration of action.
- MOR activation, decreased cAMP, and increased serotonin levels contribute to the analgesic effect, while dopamine levels remain unaffected.
- Compare to morphine, alkaloid and crude extracts of kratom cause less severe withdrawal symptoms.
- MOR activation, increased cAMP, elevated serotonin and dopamine levels contribute to the emergence of withdrawal symptoms.
- In morphine-dependent mice, treatment with alkaloid and crude extracts of kratom increased serotonin levels but decreased dopamine levels.

Keywords Alkaloid kratom, Morphine, Analgesic, Withdrawal symptoms, Serotonin, Dopamine

Introduction

Opioid drugs are routinely used to treat acute and chronic severe pain [1]. They effective reduce pain because opioids activate the opioidergic system by binding to the μu-opioid receptor (MOR) [2, 3]. This system is located throughout the peripheral and central nervous system and is responsible for modulating antinociception (analgesia) and other behavioural states such as anxiety, depression, and drug abuse [4–7]. Opioids alleviate pain by activating descending pain inhibitory circuits, which suppress nociceptive signal [8, 9].

The descending pain pathway consists of midbrain and brainstem regions that project to the dorsal horn of the spinal cord and modulate pain signals, either enhancing or inhibiting pain [10]. Serotonin is a monoamine neurotransmitter that plays a role in pain modulation via the descending pain pathway [11, 12]. Found in the raphe nuclei of the midbrain and brainstem, serotonin can either facilitate or inhibit pain depending on the receptor activated [8, 13]. Opioid drugs stimulate the release of extracellular serotonin in many brain region [9, 14]. The most widely distributed serotonin receptor, 5-HT1 A (5-Hydroxytryptamine receptor subtype 1 A), is presynaptically located on serotonergic neurons in the raphe region. This receptor sends descending serotonergic projections to the spinal cord [15, 16]. Increased serotonin at postsynaptic 5-HT1 A receptors in the dorsal horn of spinal cord inhibits the release of nociceptive neurotransmitters from sensory neurons, resulting in analgesia [9, 17].

Although opioids are effective painkillers, prolonged use of morphine and other opioid drugs leads to tolerance [18] and hyperalgesia [19, 20]. Other side effects include respiratory depression [21], constipation [22], and drug abuse [23]. MOR activation is linked to drug addiction because MOR agonists activate the dopamine reward pathway, causing euphoria and reinforcing drug abuse [24]. Activation of 5-HT1 A receptors can regulate dopamine neurotransmission and reduce the rewarding effects of drug of abuse [25, 26]. As such, understanding the mechanism of serotonin regulation is critical for managing pain and reward systems during opioid agonist therapy, as serotonin may mitigate the negative effects of increased dopamine levels.

The challenge of managing opioid withdrawal symptoms, which stem from long-term MOR activation, necessitates exploring alternative therapies. Recent studies have explored other agents that modulate pain and reward pathways differently. One of particular interest is kratom (Mitragyna speciosa), an indigenous plant grown in Southeast Asia [27]. This plant has recently garnered interest for its potential to alleviate pain with fewer adverse effects than traditional opioids [28–30]. Studies in animal models have revealed that kratom's analgesic effects are associated with its active alkaloid compounds, such as mitragynine and its derivatives [31–34]. Several in vivo pharmacological studies have been shown that mitragynine exerts antinociceptive effects through the activation of supraspinal opioid receptors mediated by MOR [35, 36]. Additionally, mitragynine has demonstrated potential in mitigating opioid withdrawal symptoms [37–40], as it may help physically dependent individuals transition away from opioids with less severe withdrawal symptoms [39, 40]. Recent studies indicate that kratom alkaloids extract induce less severe withdrawal symptoms in mice. Treatment with kratom alkaloid extract, as well as natural and semi-synthetic mitragynine, ameliorates withdrawal symptoms in morphine-dependent mice [41, 42].

Kratom alkaloids are renowned for their ability to bind a wide range of peripheral and central nervous system receptors [43], including serotonin receptors [44, 45]. Kratom's alkaloids, particularly mitragynine, have shown efficacy in reducing opioid withdrawal symptoms [38, 42]; however the serotonergic mechanisms underlying these effects remain poorly understood. Additionally, preclinical animal studies have indicated that kratom extract mitigates withdrawal symptoms in morphine-dependent rats and produces analgesic effects comparable to

conventional opioids, highlighting the role of serotonergic pathways in kratom's mechanism of action [44, 45]. Therefore, the present study aimed to determine whether central serotonin transmission, which has been shown to produce analgesic effects via the opioidergic system, contributes to the development of withdrawal symptoms in mice receiving chronic and escalating doses of kratom extract therapy. Furthermore, this study also evaluated how serotonin release induced by kratom extract treatment affected withdrawal symptoms in morphine-dependent mice. This study addresses a gap in previous studies [45, 46] by exploring kratom's potential to modulate both the serotonergic and opioidergic systems, focusing on how these mechanisms influence both analgesia and withdrawal symptoms. Unlike other studies that primarily examine MOR-specific mechanisms, this research broadens the scope of investigation.

Materials and methods

Animals

Male BALB/c mice (25–30 g) used in this study were obtained from PT Biomedical Technology Indonesia (Bogor, Indonesia). The mice were housed at 22–25 °C, humidity 50–70% with free access to water and food (ad libitum) under a 12 h/12 h light–dark cycle. Before the experiment, they underwent a one-week acclimatisation period in the laboratory, during which they were handled daily to become accustomed to the experimenter. All experimental procedures, including the treatment of animals, were reviewed and approved by the Ethical Committee on Health of the National Research and Innovation Agency, Republic of Indonesia (053/KE.03/SK/05/2023).

Plant materials

Plant material collection was conducted in accordance with the World Health Organization's Quality Control Methods for Medicinal Plant Materials (1998) and was approved by the Research Organization for Health, National Research and Innovation Agency (BRIN), Republic of Indonesia. Fresh kratom leaves were collected from a kratom plantation in Kapuas Hulu, West Borneo, Indonesia. Our institution (BRIN) obtained permission to collect samples based on a mandate from the Chief of Staff of the Presidency of the Republic of Indonesia to conduct a study on the kratom plant to obtain scientific evidence as a basis for policy-making by the Indonesian government regarding the status of the kratom plant. The plant was identified as *Mitragyna speciosa Korth.* by Wihermanto, a biological collections curator from the Directorate of Scientific Collection Management, BRIN. The specimen of this plant had also been deposited at Herbarium Bogoriense, BRIN with deposition number BO-1328479. Briefly, the leaves were sun-dried until their moisture content fell below 10%. Afterward, the dried leaves were pulverized and filtered through a 0.5-mm mesh filter under good manufacturing practices. The powdered leaves were stored in a sealed plastic bag at 4 °C for further examination. The extract was prepared following our protocol [47, 48], yielding two extracts; *i.e.* kratom methanolic crude extract and alkaloid extract.

Chromatographic analysis

The quantitative analysis was conducted using liquid chromatography following our protocol [47]. Mass spectrometry analysis was performed according to another study [49] with a slight modification. In brief, a stock solution of each extract was prepared at 10 mg/mL in methanol (LC-grade). The solutions were filtered through 0.22 µm nylon membrane syringe filter before being injected (10 µL) into the Thermoscientific Orbitrap Exploris 120®, equipped with a heated electrospray ionization (ESI) source. Separation was done using a ZORBAX EclipsePlus C18 RRHD column (2.1 mm × 100 mm × 1.8 µm) at 35°C. Mobile phase A consisted of 0.1% formic acid in water, while mobile phase B consisted of 0.1% formic acid in acetonitrile. Elution was set to gradient mode (Table 1). The mass spectrometer was set with a scan range at 100–1000 m/z.

Preparation for the drugs

Morphine hydrochloride (batch number 310216, PPPOMN BPOM, Indonesia) and naloxone hydrochloride (batch number B0120414, PPPOMN BPOM, Indonesia) were provided by the Indonesian Food and Drug Authority (BPOM). In brief, each compound was diluted in water (Otsuka, Indonesia) and administrated at doses of 10 mg/kg and 3 mg/kg, respectively [33]. The crude and alkaloid extracts of kratom were dissolved in a vehicle consisting of 20% Tween-80 and

Table 1 Elution condition for mass spectrometry analysis using LC–MS equipped with Orbitrap detector

Time (min)	Eluent A (%)	Eluent B (%)	Flow rate (mL/min)
0.00–5.00	98	2	0.5
5.00–35.00	76	24	0.5
35.01–42.00	98	2	0.4
42.00–58.00	20	80	0.4
58.00–59.00	20	80	0.4
59.00–67.00	98	2	0.4

distilled water. The final solution was administered at a dose of 10 mg/kg for the crude extract and 20 mg/kg for both the crude and alkaloid extracts.

Analgesic hot-plate test and tolerance induction

The analgesic effect was evaluated using the hot-plate test method [34], with six mice in each groups. The groups included the control (vehicle), morphine-treated (10 mg/kg), two groups treated with kratom crude extract (10 mg/kg and 20 mg/kg), and a group treated with kratom alkaloid extract (20 mg/kg) (Fig. 1). A single dose of alkaloid extract was set at 20 mg/kg to achieve a mitragynine content (~ 46%) comparable to morphine alone. On the other hand, the crude extract dosage of 20 mg/kg was selected to match the same dose level as those samples, while the comparison was also conducted at a lower level (10 mg/kg). The mice were treated intraperitoneally (ip.) twice a day for ten days (Fig. 1A).

The hot-plate test involved placing each mouse in a 15 cm diameter plexiglass cylinder on a hot-plate set to 55 °C and measuring the latency period before the mice displayed nociceptive responses such as licking their hindlimbs (Figure S1). The pre-drug latency was determined by measuring each mouse's nociceptive baseline three times before the testing day. A cut-off time 45 s was established to minimize tissue damage. The analgesic effect of the treatments was measured daily in the morning at intervals of approximately 15 min (15, 30, 45, 60, 90, 120, 150, and 180). The maximum possible effect (MPE) of analgesia effect was determined for each day, representing an average over the eight observation time points, and was calculated using the following formula:

$$\text{MPE (\%)} = \frac{\text{Post drug latency - Pre drug latency}}{\text{Cut-off time - Pre drug latency}} \text{ x } 100$$

Naloxone-precipitated withdrawal assay

Mice were administered either morphine, crude extract, alkaloid extract, or vehicle intraperitoneally (ip.) twice daily (morning and evening). The dose was gradually increased from 8 to 45 mg/kg over five days (Table 2, Fig. 2A). Naloxone (3 mg/kg) was administered two hours after the final treatment dose, as previously described [33]. The mice were immediately placed in a plexiglass

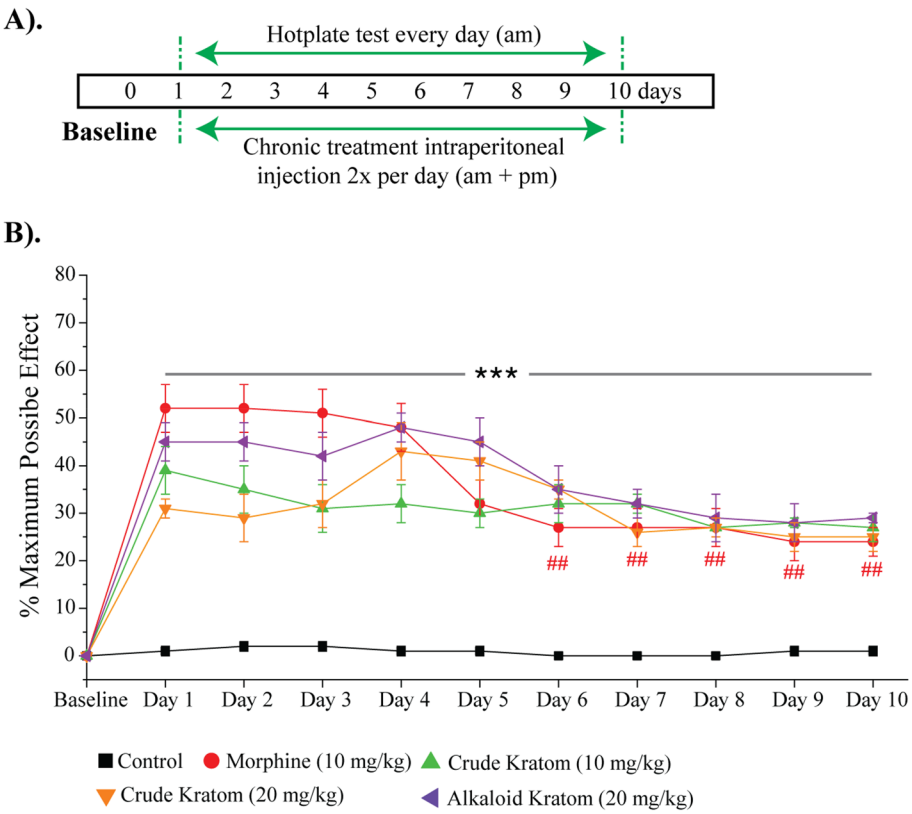

Fig. 1 Development of tolerance to analgesic effects for acute thermal pain in paw hot-plate test. **A** Schematic diagram for chronic treatment of vehicle, morphine, crude extract and alkaloid extract in a hot-plate test over ten days. **B** Percent maximum possible effect (% MPE) from day one until day ten. *** $p < 0.001$ significantly different from control groups; ## $p < 0.01$ significantly different from day one administration of morphine, one-way ANOVA with Tukey's post hoc test

Table 2 Escalating dose scheme over 5 days to evaluate potential withdrawal symptoms

Days	Doses (mg/kg)	
	Morning (AM)	Afternoon (PM)
1	8	15
2	20	25
3	30	35
4	40	45
5	45	-

An escalating dose was used for morphine, kratom crude extract, and kratom alkaloid extract

cylinder, and their withdrawal symptoms were observed for 30 min (Table 3).

Another group of animals received an escalating dose of morphine following the same protocol (Table S3, Fig. 3A). On days 6–8, the three morphine groups were administered saline, crude extract, or alkaloid extract (10 mg/kg) twice daily. On day 8, after the naloxone challenge, the mice were placed in a plexiglass cylinder for withdrawal symptom observation.

Withdrawal symptoms precipitated by naloxone were assessed using the same method as in morphine-treated animals [41, 42, 50, 51]. Jumping behaviour was quantified over a 30-min observation period. Meanwhile, symptoms such as ptosis, straightening, and diarrhea were evaluated at 5-min intervals. Each sign was assigned a score of 1 for each interval in which it was observed, up to the 30-min observation time. The global withdrawal score (GWS) was calculated by multiplying the total for each sign by a constant (weighing factor) and then summing the scores for each sign [2, 50–52]. The GWS determines the severity of withdrawal symptoms.

Determination of MOR, cyclic adenosine monophosphate (cAMP), serotonin (5-HT), dopamine (DA) expression in the brain

Following behavioural experiments, mice were sacrificed using a surgical plane of anaesthesia (ketamine 100 mg/kg and xylazine 10 mg/kg, ip. 100 mg/kg) followed by perfusion

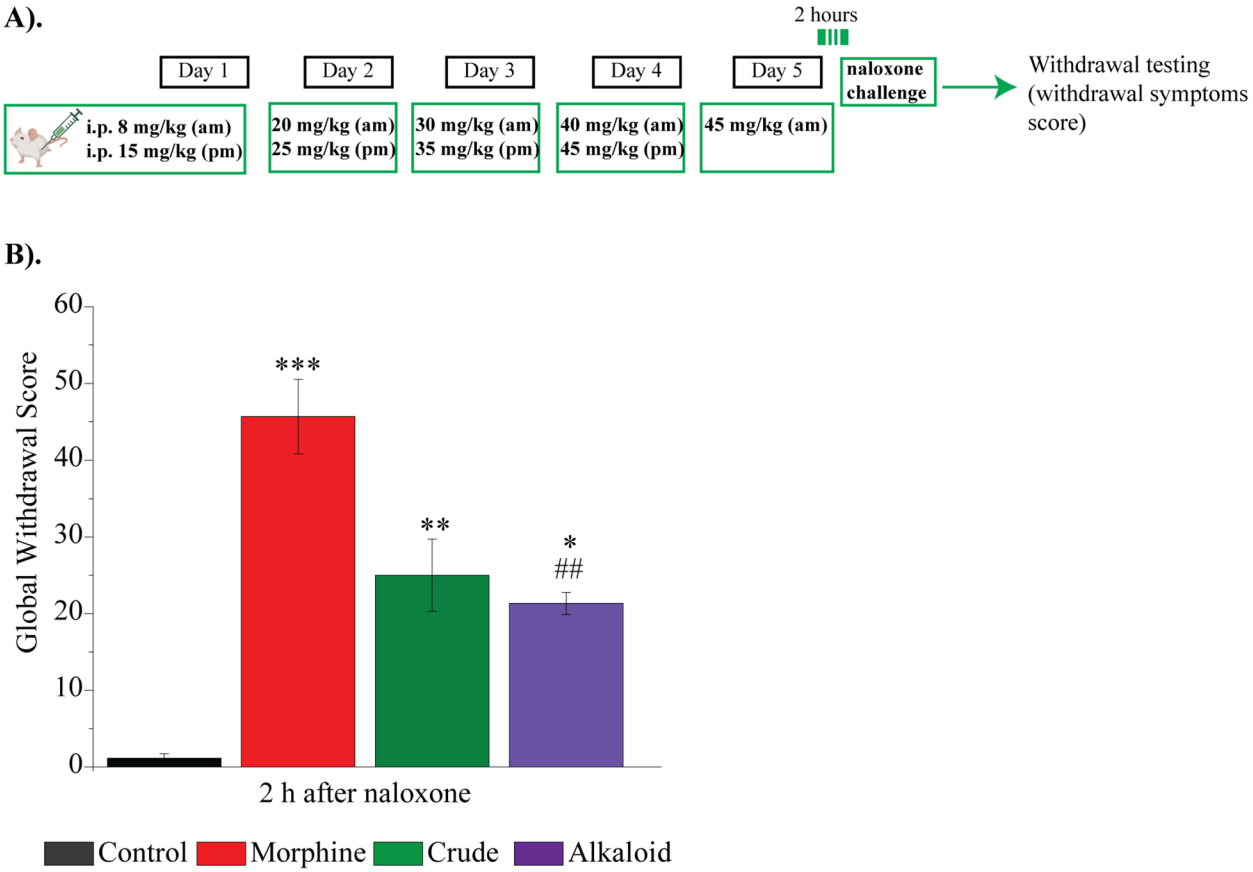

Fig. 2 Naloxone induced withdrawal symptoms following chronic and escalating dose of morphine and kratom extracts. **A** A schematic diagram of chronic doses of morphine or kratom extracts administered over 5 days to cause naloxone-induced withdrawal symptoms. **B** Global withdrawal score for all treated-mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ significantly different from control groups; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ significantly different from morphine groups, one-way ANOVA with Tukey's post hoc test

Table 3 Summary of behaviours observed during naloxone-precipitated withdrawal period

Behaviour	Weighing factor	Description
Jumping	2	All four paws are off the ground
Ptosis	2	Drooping of the upper eyelids
Straightening	2	Number of times when the mice stretched their body with all four paws touching on the ground
Diarrhea	1	Number of soft and or wet faeces

via cardiac puncture. Their brains were then collected in RNase-free Eppendorf tubes containing 1 ml of cold RIPA buffer (Sigma Aldrich, USA) as a lysis buffer. The brain was crushed and homogenized via ultrasonication for 5 s. After homogenization, the samples were refrigerated at −80 °C until use. The expression levels of MOR, cAMP, 5-HT, and DA in the brain tissue homogenates were quantitatively analyzed using an Enzyme-Linked Immunosorbent Assay (ELISA) kit (Elabscience, China) according to the manufacturer's protocol. The following ELISA kit were used in this study: mouse DA Elisa kit (E-EL-0046, Lot DP01 F2RX6283), mouse 5-HT/ST ELISA kit (E-EL-0033, Lot WZ09 V6801220), mouse cAMP ELISA kit (E-EL-0056, Lot WZ074 T6 T7937), mouse Oprm1(mu-type opoid receptor) ELISA kit (Fine Test, China, EM2514).

Molecular docking and molecular dynamics

Molecular docking was performed on tryptophan hydroxylase (TPH). The structure of TPH (PDB ID: 1MLW) was obtained from the RCSB Protein availabilityBank (PDB). The TPH structure was prepared using Autodok Tools version 1.5.7, which involved removing water molecules, adding polar hydrogen atoms, and

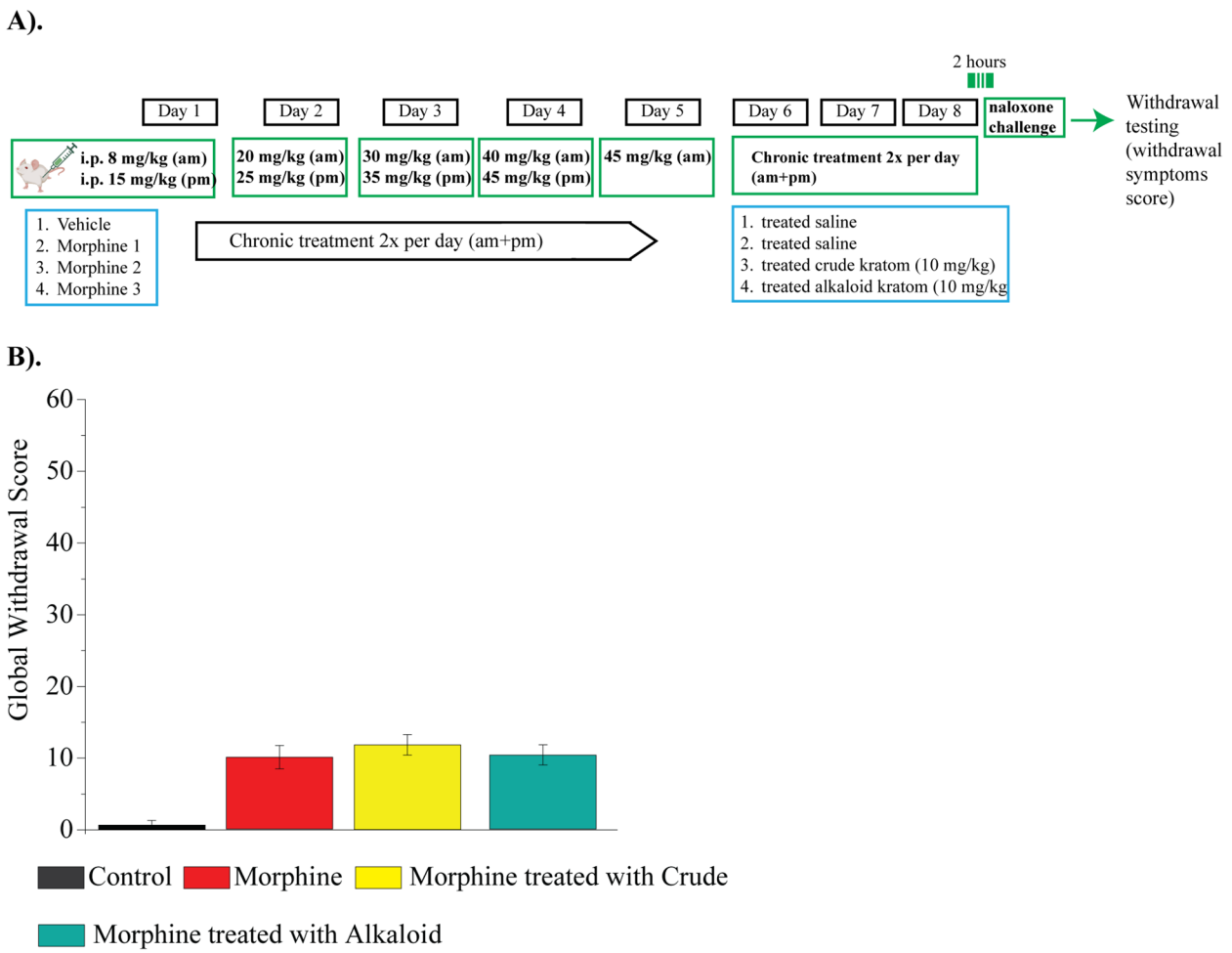

Fig. 3 Kratom extracts treatment following chronic and escalating dose of morphine. **A** Schematic diagram of naloxone-induced withdrawal symptoms in all treatment with morphine groups as positive control groups. **B** Global withdrawal score for all treated-mice alongside morphine-saline treatment and vehicle treatment, one-way ANOVA with Tukey's post hoc test

assigning Gasteiger charges. The natural co-factor of TPH, 7,8-dihydrobiopterin (HBI), and the Fe ion were removed to isolate the TPH structure. Six alkaloids from *Mitragyna speciosa* were selected as ligands based on the result of our mass spectrometry analysis (Figure S4), and their 3D structures were obtained from the PubChem database in.sdf format. Ligand preparation was performed using RDKit and Open Babel v3.1.1, which included energy minimization, optimization, and conversion of the.sdf file to.pdbqt format. Hydrogen atoms and charges were added using AutoDock Tools, and the ligands were split into individual files using AUtoDock Vina commands.

Molecular dynamics simulations were performed using a combination of CHARMM-GUI and GROMACS tools. The simulation was conducted for 100 ns with the AMBER14 FF parameter set at a temperature of 310 K, pH 7.0, and NaCl ion concentration of 0.15%. The topology preparation for the GROMACS MD simulation was carried out using the CHARMM-GUI web server with the solution builder feature. The preparation stages included reading the protein and ligand PDB files, minimization, and equilibration. The GROMACS MD simulation was executed using a CPU-based MPI, following three stages: minimization, equilibration, and final MD simulation.

Statistical analysis

The data are expressed as the mean ± standard error of the mean (SE). The statistical analysis and all graphs were performed using OriginPro 2018 software (OriginLab, Japan). The behavioural data and ELISA data for protein expressions were analysed using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, with $p < 0.05$ considered significant.

Results

Kratom extracts exert analgesic effects in the hot-plate test

The analgesic effects of treatment for acute thermal pain are determined by the percentage of maximum possible effects (% MPE) in the hot-plate test (Fig 1B). Treatment with alkaloid extract (20 mg/kg) produced an analgesic effect comparable to that of morphine (10 mg/kg). The morphine group exhibited the highest MPE on day 1, while the alkaloid group had the highest MPE on day 4, at 52 ± 5% and 48 ± 3%, respectively. The crude extracts also induced analgesia, albeit at a slightly lower % MPE (Day 4 for crude 20 mg/kg with highest %MPE at 43 ± 6% and Day 1 for crude 10 mg/kg with highest %MPE at 45 ± 3%) compared to the morphine group. Furthermore, repeated morphine treatment decreased its effectiveness by approximately 56% after 6 days. This finding is consistent with previous studies that have shown morphine tolerance after 5 days [33]. Interestingly, alkaloid extract treatment reduced analgesic effects only after 10 days. This result suggests that alkaloid extract provides an equivalent analgesic effect but with longer-lasting efficacy compare to morphine group.

To determine which pathway is responsible for the analgesic effect of kratom extracts, mice were sacrificed, and their brains were examined for protein content. Morphine treatment (10 mg/kg) for acute thermal pain significantly increased MOR levels compared to the control group (MOR level = 452 ± 42.8 pg/mL; $p < 0.001$; Fig. 4A). Similar to morphine, both crude and alkaloid kratom extracts could enhanced MOR levels (MOR level for crude extract 20 mg/kg = 381.7 ± 13.8 pg/mL; MOR level for alkaloid extract 20 mg/kg = 379.7 ± 30 pg/mL $p < 0.05$; Fig. 4A). Meanwhile, crude extract at 10 mg/kg had lower MOR levels compared to the other treatments (MOR level for crude extract 10 = 329.9 ± 42.8 pg/mL $p < 0.05$; Fig. 4A). Activation of MOR for analgesic effects lowered intracellular cAMP levels in all treatments compared to the control group ($p < 0.01$; Fig. 4B).

Next, we examined the neurotransmitter involved in the analgesia effect via MOR activation. No significant difference in dopamine levels was observed during analgesia, except for the kratom crude extract at 20 mg/kg, which showed a significant increase (50 ± 2.5 ng/mL, $p < 0.001$; Fig. 4C). Meanwhile, all treatments increased serotonin levels, with morphine and crude extract 10 mg/kg showing higher levels than control group (serotonin level in the morphine group = 265 ± 15 ng/mL, $p < 0.001$; kratom crude extract 10 mg/kg = 289.6 ± 20 ng/mL, $p < 0.001$; Fig. 4D).

Naloxone induced withdrawal symptoms in kratom extracts-treated mice

Another group of mice received chronic and escalating doses of kratom extracts (Table 2), followed by naloxone treatment (Fig. 2A), to determine whether they exhibited the same withdrawal symptoms as those induced by chronic opioid use [33]. Withdrawal symptoms observed in rodents include increased jumping, self-directed behaviours (such as increased grooming and penile licking), hyperactivity (including locomotion, digging, and rearing), vocalization, ptosis, wet dog shakes, teeth chattering, and gastrointestinal motility (manifested as increased defecation and diarrhea) [53]. In our study, we observed jumping, ptosis, straightening, and diarrhea frequency, all of which were significantly different from those in the control group (Table 3–4). In general, morphine treatment induced more severe withdrawal symptoms compared to those treated with crude or alkaloid extracts (Fig. 2B). Mice treated with morphine showed a global withdrawal

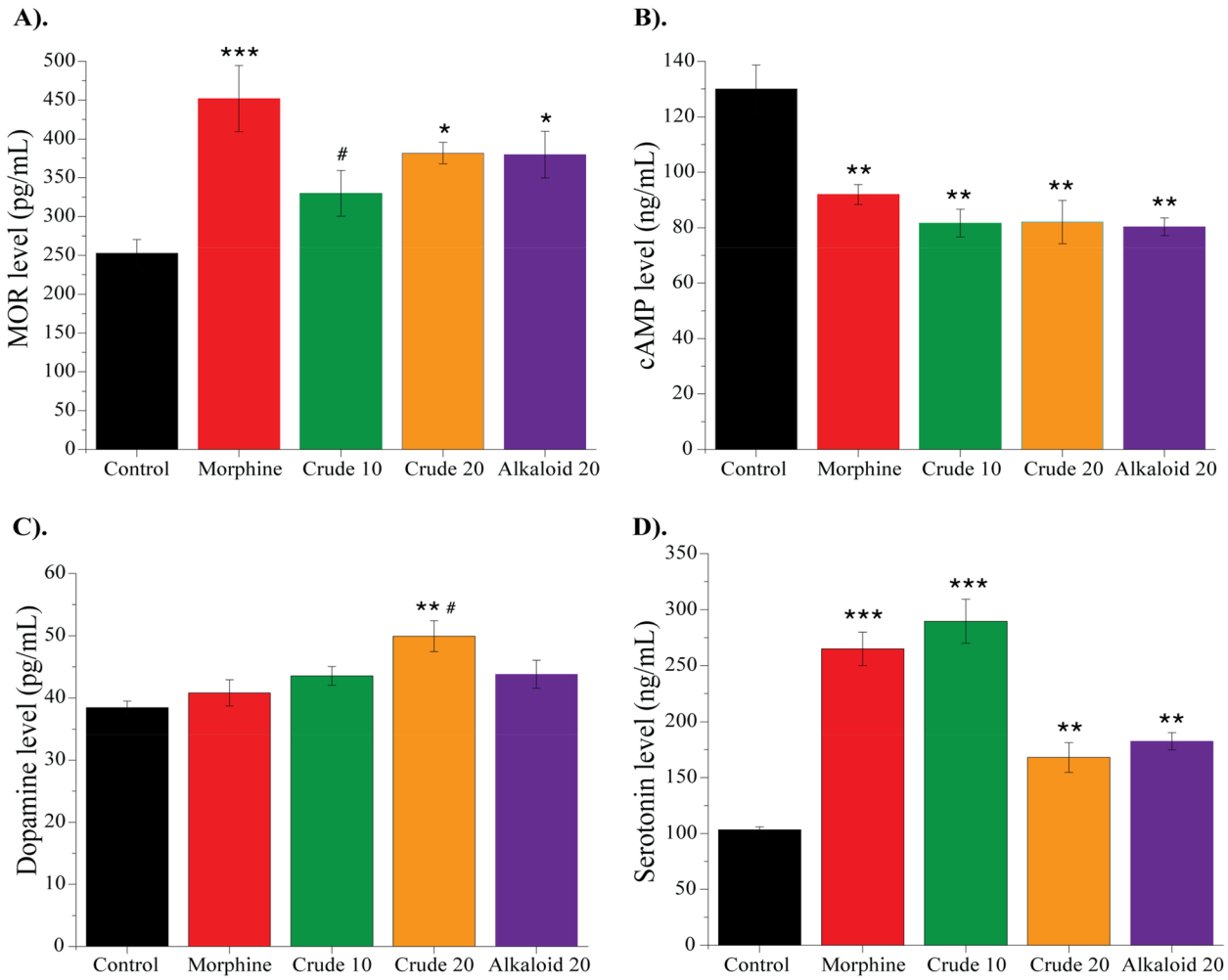

Fig. 4 Analgesic effects of morphine and kratom extracts on MOR, cAMP, serotonin and dopamine levels measured using ELISA kit. Following the fourth-day of chronic treatment, mice were sacrifice, and the brain was collected to determine the protein expression levels for **A**) MOR, **B**) cAMP, **C**) dopamine, and **D**) serotonin level in the brain which may responsible for the analgesic effects. *** $p < 0.001$, ** $p < 0.01$ significantly different from control groups; # $p < 0.05$ significantly different from morphine groups, one-way ANOVA with Tukey's post hoc test

Table 4 Average withdrawal score for each behavioural symptoms observed 2 h after naloxone

Withdrawal Symptoms	Control	Morphine	Kratom crude extract	Alkaloid Extract
Ptosis	0.8 ± 0.37	7.9 ± 0.97 ***	7.6 ± 1.52 **	8.9 ± 1.21***
Jumping	0.3 ± 0.25	22.2 ± 4.55 **	4.9 ± 3.25 #	0.4 ± 0.29 #
Straightening	0.3 ± 0.25	2.6 ± 0.59	4.4 ± 1.14 *	6.9 ± 1.53 ***,##
Diarrhea	0.3 ± 0.25	1.1 ± 0.30	0.6 ± 0.18	0.8 ± 0.15

* $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$ significantly different compared to the control

# $p < 0.05$, ## $p < 0.01$ significantly different compared to the morphine group

score (GWS) of 46 ± 4.8 ($p < 0.001$). In contrast, mice treated with crude and alkaloid extracts experienced less severe withdrawal symptoms than the morphine-treated groups (Fig. 2B), with lower GWS values of 25 ± 4.7 and 21 ± 1.5, respectively.

Morphine, as well as both crude and alkaloid extracts, activated MOR, leading to withdrawal symptoms (Fig. 5A). Our study found that mice treated with crude and alkaloid extracts experienced withdrawal symptoms and increased cAMP levels similar to those in the

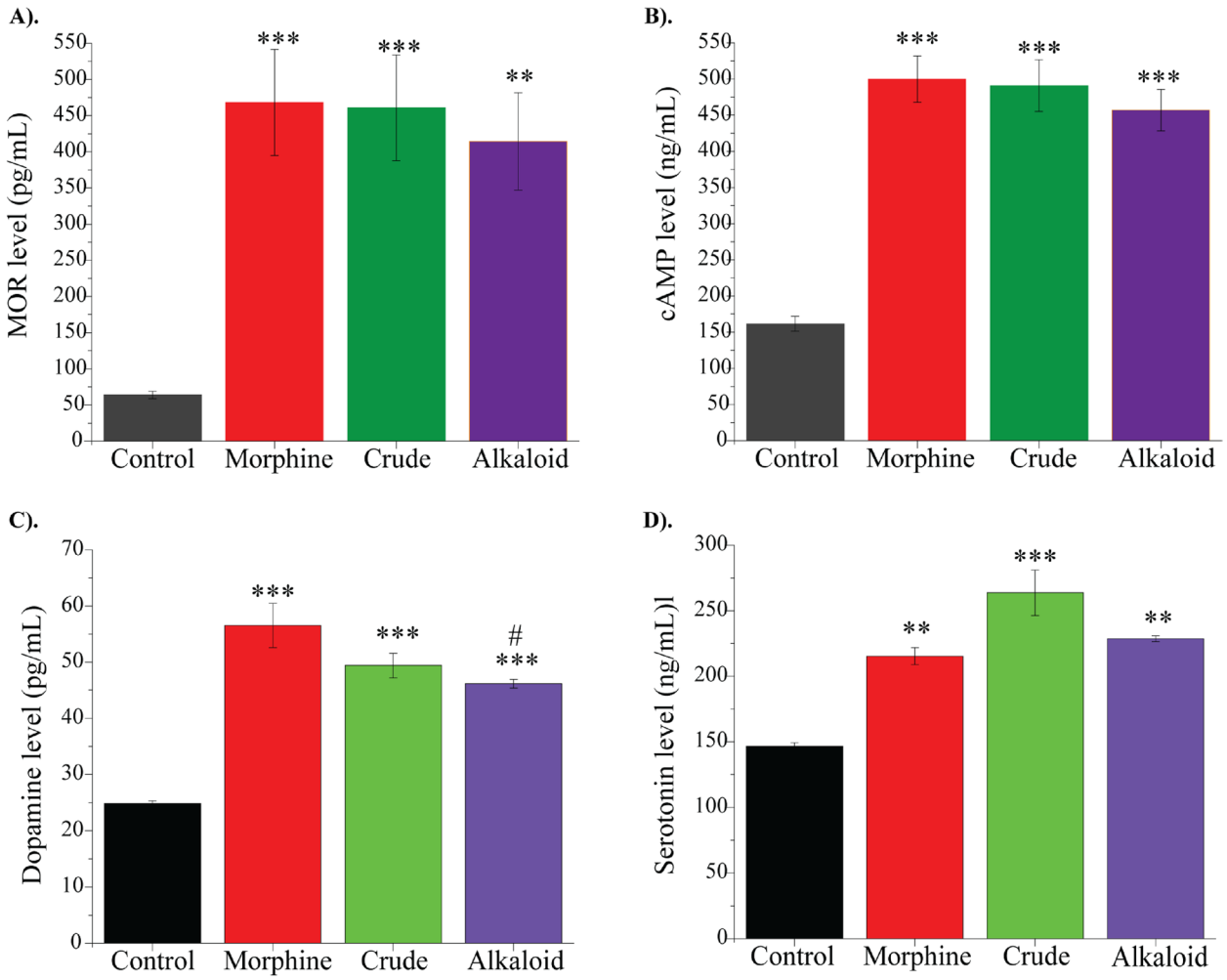

Fig. 5 Naloxone precipitated withdrawal symptoms after chronic morphine and kratom extracts treatment on MOR, cAMP, serotonin and dopamine levels measured using ELISA kit. On the fifth-day of chronic treatment, mice were injected with naloxone and sacrificed an hour later. The brain was then collected to determine the protein expression levels for **A**) MOR, **B**) cAMP, **C**) serotonin, and **D**) dopamine level in the brain which may responsible for the emergence of withdrawal symptoms. *** $p < 0.001$, ** $p < 0.01$ significantly different from control groups; # $p < 0.05$ significantly different from morphine groups, one-way ANOVA with Tukey's post hoc test

morphine-treated groups ($p < 0.001$; Fig. 5B). Two hours after the final dose, morphine and both kratom extracts continued to raise dopamine levels in mice that experienced withdrawal symptoms following naloxone administration ($p < 0.001$ compared to control; Fig. 5C). In addition to dopamine, the mice exhibiting withdrawal symptoms also had significantly higher serotonin levels ($p < 0.001$ compared to control; Fig. 5D).

Opioid withdrawal treatment using kratom extracts

We investigated the reduction of withdrawal symptoms effect on morphine group treated with either alkaloid extract (10 mg/kg) or kratom crude extract (10 mg/kg) (Fig 3). The withdrawal symptoms were barely visible after three days of treatment, as they are with lower GWS (Table 5). The serotonin level remained high compared to control in all treatment group ($p < 0.01$), even when the withdrawal symptoms almost diminished in all morphine group treated with saline or crude and alkaloid extracts. Interestingly, the dopamine level was decreased in all morphine group treated with kratom extracts ($p < 0.01$) (Fig. 6).

In silico analysis of alkaloid kratom and enzyme tryptophan hydroxylase

Molecular docking was conducted to examine the binding affinity between tryptophan hydroxylase (TPH) and kratom alkaloid extract. TPH is an enzyme that catalyses the rate-limiting step in serotonin synthesis [54–57]. The alkaloids contents in this study were analysed using

Table 5 Withdrawal score for each behavioural symptoms observed after 3 days kratom extract treatment

Withdrawal Symptoms	Control	Morphine-Saline treatment	Morphine-Kratom crude extract treatment	Morphine-Alkaloid treatment
Ptosis	0	5.2 ± 2.25	7.6 ± 1.6 *	6.7 ± 0.98 *
Jumping	0	0	0	0
Straightening	0	0.8 ± 0.8	3.6 ± 1.8	3 ± 0.86

* $p < 0.05$ significantly different compared to the control

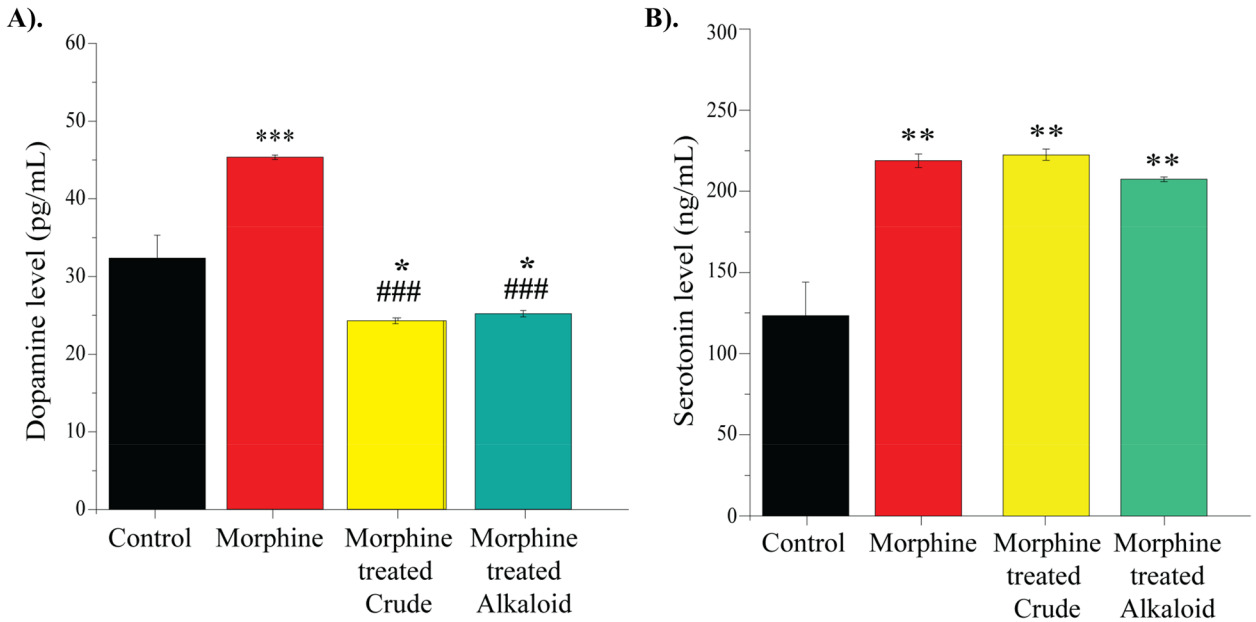

Fig. 6 Serotonin and dopamine levels in the morphine group treated with kratom extracts measured using ELISA kit. On the third-day of treatment, morphine treated mice were injected with naloxone and sacrificed an hour later. The brain was then collected to determine the protein expression levels for **A**) dopamine, and **B**) serotonin after treatment in group of mice previously treated with morphine for consecutive five days. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ significantly different from control groups; # $p < 0.05$ significantly different from morphine groups, one-way ANOVA with Tukey's post hoc test

mass spectrometry (Table S2) and their chromatographic profiles were further confirmed by other studies (Figure S2-S8) [58–62]. Critical residues for binding, such as His251, Tyr235, His277, Tyr312, Ala309, and Phe24 on chain A, were identified from crystallographic data (1MLW; https://www.rcsb.org/structure/1MLW) (Fig. 7A-C; Table S3-S5). Of particular interest, the alkaloid speciociliatine of *Mitragyna speciosa* showed the highest binding affinity of −9.0 kcal/mol, surpassing both controls and five other alkaloids (Table S4), in stabilizing the binding pocket similarly to HBI (7,8-dihydrobiopterin).

Molecular dynamics simulations were performed for three complexes: native ligand HBI-TPH, morphine-TPH, and speciociliatine-TPH, to provide data for their specific binding and interaction stability (Fig. 7D-G). Based on root mean square deviation (RMSD) and root mean square fluctuation (RMSF) analyses, the native ligand HBI, morphine and speciociliatine exhibited similar graphical patterns, with RMSD and RMSF values averaging below 2 Å. The radius of gyration values ranged between 1.83–1.88 nm, indicating that the interaction between the protein and ligand was relatively stable. The final parameter evaluated was protein–ligand stability based on hydrogen bond interactions. The results showed slight differences, with HBI forming more hydrogen bonds than morphine and speciociliatine. This suggests variations in hydrogen bond interaction intervals; however, they are expected to have similar effects.

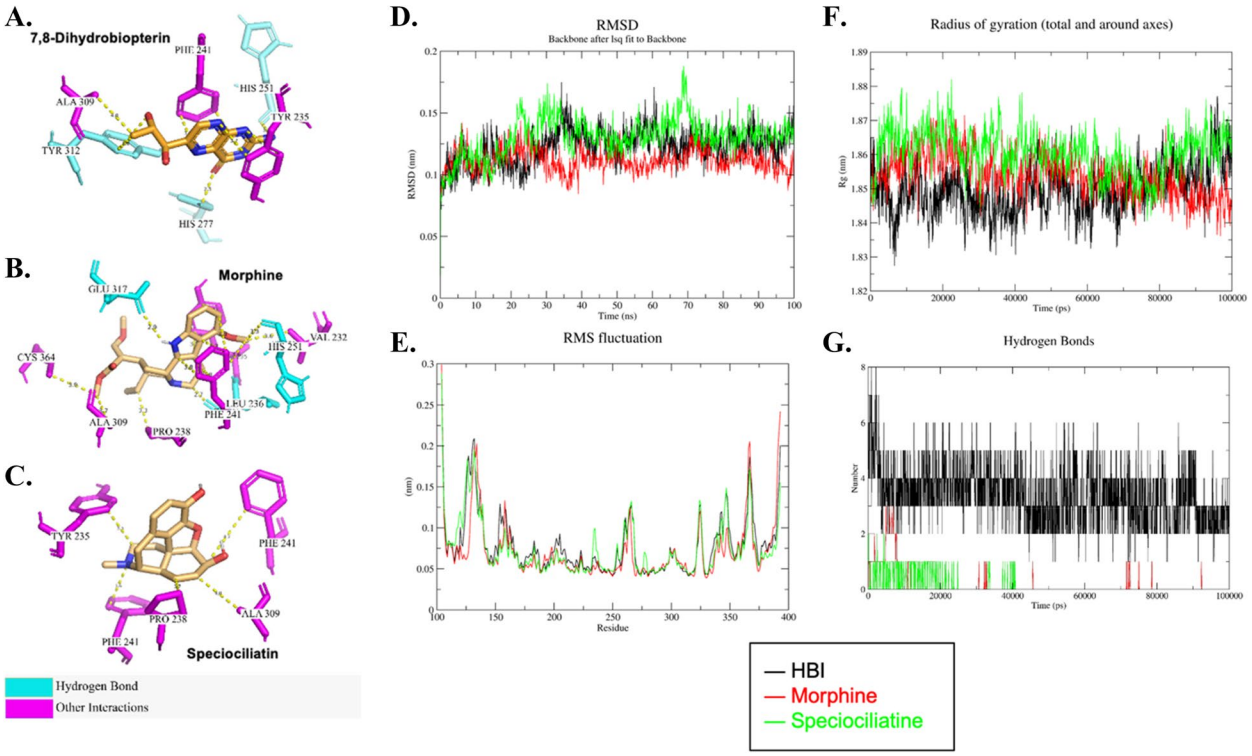

Fig. 7 Molecular Docking and Molecular Dynamics Simulation of TPH-Ligand Interactions. Docking interactions between tryptophan hydroxylase (TPH) and its ligands; (**A**) native cofactor 7,8-Dihydrobiopterin (HBI), (**B**) morphine, and (**C**) speciociliatine**.** Hydrogen bonds **(**cyan**)** and other interactions **(**magenta**)** are highlighted. Molecular dynamics (MD) analysis over 100 ns: RMSD shows complex stability (**D**), RMS Fluctuation indicates residue flexibility (**E**), and radius of gyration (Rg) reflects protein compactness (**F**). HBI (black), morphine (red), and speciociliatine (green) show comparable stability. (**G**) Hydrogen bond analysis**,** with HBI forming the most bonds, followed by morphine and speciociliatine

Discussion

Kratom alkaloid binds to the MOR and induces analgesia [28, 44, 63]. We discovered that kratom extracts, like morphine, increased MOR concentration, mediating analgesic effects on heat-stimulus pain (Fig. 4A). When a μu-opioid agonist binds to the MOR, a series of molecular cascades may be activated, including a decrease in cyclic adenosine monophosphate (cAMP) levels as an immediate cellular event [64–66]. Previous in vitro studies have shown that the addition of morphine lowers intracellular cAMP levels [67, 68]. Although the exact mechanism through which these signals affect analgesia remains unclear, opioid receptor-mediated cAMP signalling may contribute to pain relief [69, 70]. Our results confirmed that mice exhibiting analgesic effects had lower cAMP levels (Fig. 4B). However, continued exposure to morphine increases adenylyl cyclase and intracellular cAMP levels. When morphine is eliminated from the body, cAMP concentrations rise above pre-morphine levels [67, 68]. Our data also revealed that abrupt discontinuation of chronic treatment with morphine and kratom extracts significantly increased intracellular cAMP levels above those in the control group (Fig. 5B).

Monoamines neurotransmitter, including norepinephrine, serotonin, and dopamine, play a role in regulating the endogenous pain system [71]. It is known that neurotransmitter serotonin in the central nervous system is essential for opioid-induced analgesia [72]. In vivo studies indicate that activating the postsynaptic 5-HT1 A receptor subtype in the dorsal horn of the spinal cord inhibits glutamate release, thereby reducing pain transmission [9, 17]. Our acute thermal pain paradigm revealed that serotonin release was increase when mice exhibited analgesic effects (Fig. 4D). Activating opioid receptors weakens the nociceptive signal while it increases dopamine neurotransmission [73, 74]. However, our findings suggest that dopamine release is less effective than serotonin release in mediating analgesia (Fig. 4C). This could be because the transient nature of phasic pain stimuli (sharp and short lasting sensation) in tests such as the tail flick, hot plate, or paw pressure assays, which may not significantly engage the dopaminergic system. In contrast, tonic pain assays using formalin or writhing tests, activate D2-dopamine receptors, resulting in greater pain relief [75, 76]. Other studies have reported no significant difference in response to dopaminergic modulation across various

pain tests [77–79]. Dopamine release appears to be more active during the motivational-emotional component of the pain test, such as conditioned place preference for pain relief, rather than directly mediating analgesic effects [76, 80].

Repeated opioid exposure may cause tolerance, as could be seen by a 56% decrease in analgesic efficacy after six days of morphine treatment. Interestingly, treatment with the alkaloid extract only reduced the analgesic effect after ten days of chronic treatment (Fig. 1B). This is probably due to the delayed pharmacokinetic of alkaloid kratoms. Previous study have confirmed that mitragynine has slow drug clearance but high efficacy in crossing the blood–brain barrier [81, 82]. Prolonged opioid use can also lead to physical dependence, marked by withdrawal symptoms within hours of discontinuation [53]. As an opioid antagonist, naloxone, is used to treat opioid overdose [83]; however, administrating naloxone too soon after chronic opioid use can trigger unpleasant withdrawal symptoms [33, 53]. Chronic and increasing doses of kratom extracts, followed by abrupt discontinuation and naloxone treatment, produce withdrawal symptoms comparable to those observed in morphine-dependent subjects. However, the GWS score was lower in kratom extract-dependent mice than in morphine-dependent mice (Fig. 2B, Table 4). Other studies have found that treatment with kratom alkaloid, such as mitragynine, or mitragynine pseudoindoxyl, results in fewer withdrawal symptoms [30, 40, 41]. Most kratom alkaloids function as a partial agonist of the MOR receptor [28, 30, 44]. Thus, it differs slightly from morphine, which is a full agonist of the MOR [44]. As a result, withdrawal symptoms seen in kratom extract-treated mice may be less severe compared to those in morphine-dependent mice.

Dopamine and serotonin are among two neurotransmitters that influence the development of opioid use disorders [84]. Our findings revealed that withdrawal symptoms were associated with increased serotonin and dopamine levels across all treatments (Fig. 5C, 5D). These findings contradict with previous studies showing that dopamine and serotonin levels decrease during withdrawal [84]. Chronic opioid use induces withdrawal symptoms, which may dampen dopamine release, leading to reward deficits and increase in negative emotions such as fear, anxiety, and stress [84, 85]. The discrepancy between our study and previous studies is likely due to the difference in the upper limit of dose range (45 mg/kg) administered chronically to induce physical dependence compared to other similar studies (morphine 70 mg/kg, and alkaloid kratom 125 mg/kg) [41, 42]. Since the mice in our study received a much lower dose to induce dependence, it is reasonable to assume that they were still in a stage where they experienced the same euphoria as after their initial exposure, reinforcing opioid use (binge/intoxication stage).

To determine whether kratom extracts could alleviate withdrawal symptoms, another group of morphine-dependent mice was treated for three days with crude and alkaloid extracts (10 mg/kg). We observed a reduction in withdrawal symptoms across all treatments, including in morphine-dependent mice treated with saline for three days. The reduced withdrawal symptoms in all groups were most likely due to the lower dose used to induce physical dependence, which resulted in milder withdrawal symptoms even without treatment with kratom extracts. Another study used higher doses of morphine (10 mg/kg–80 mg/kg) to induce physical dependence, followed by treatment with kratom alkaloids extract, which effectively alleviate morphine/opioid withdrawal symptoms [41]. Surprisingly, in our study, both kratom extract treatments in morphine-dependent mice increased serotonin levels while decreasing dopamine levels. This result aligns with previous studies suggesting that serotonin may inhibit the rewarding effects of drug abuse by modulating dopamine neurotransmission [25, 26, 86]. However, further studies are needed to confirm this finding.

The alkaloid extract demonstrated an analgesic effect comparable to that of morphine, unlike the crude extracts. There was no significant difference in the reduction of withdrawal symptoms between the morphine-dependent groups treated with crude and alkaloid extracts. As a result, alkaloid extracts may be more effective as analgesics with fewer effects in inducing withdrawal symptoms in our experimental setup. Supporting this potential, another study reported that a COVID-19 patient experienced significant improvement in physical symptoms (less pain and fatigue) after consuming kratom (3 days, 9 doses @ 2.5–2.75 g of kratom leaf powder suspended in water) compared to treatment with paracetamol (5 days, 1 g every 6 h). They also noticed that the use of ibuprofen (one of NSAIDs) could increase the risk of severe adverse events in COVID-19 patients [87]. In acute toxicity study (14 days), rats administered kratom's methanolic extract at dose 100, 500, and 1000 mg/kg did not show mortality or symptom of toxicity, whereas contrasting results were observed in rats treated with morphine (430 mg/kg) [88]. Based on those results, we postulate that the kratom extracts may offer analgesic with fewer side effects compared to opioids like morphine and NSAIDs.

The increased serotonin levels were likely responsible for both the analgesic effect and the onset of withdrawal symptoms. These findings highlight the potential role of speciociliatine in regulating serotonin biosynthesis by directly interacting with tryptophan hydroxylase (TPH),

a key enzyme involved in the rate-limiting step of serotonin production. Our in silico analysis revealed a significant binding affinity, suggesting that speciociliatine may function as a stabilizer for TPH, similar to its natural cofactor, HBI. This stabilization could enhance the enzyme's activity, leading to increased serotonin levels, a result consistent with our in vivo findings. Previous studies indicate that TPH activity is highly dependent on its structural stability and the presence of its cofactors [89]. This further supports the idea that kratom alkaloids, especially speciociliatine, may promote serotonin synthesis through direct interactions with the enzyme. Additionally, key binding residues such as His251 and Tyr235 align with known functional sites critical for TPH activity, as demonstrated in structural studies of the enzyme [89]. Given that serotonin is crucial for pain modulation and managing withdrawal symptoms, speciociliatine's ability to enhance serotonin production may explain its dual functionality in providing analgesic effects and managing withdrawal. This mechanistic insight suggests that kratom-derived alkaloids merit further exploration as novel therapeutic agents, not only for pain relief but also for alleviating withdrawal symptoms related to opioid dependence. Additional structural and functional studies, including enzyme kinetics and mutagenesis analyses, are needed to confirm these computational predictions and validate the therapeutic potential of kratom alkaloids in modulating serotonergic activity.

Conclusion

Opioid drugs such as morphine are effective for therapeutic pain due to their activity on the opioidergic system via binding to MOR. However, full activation of this system modulates antinociception and behavioural states (*e.g.*, anxiety, depression, drug abuse), resulting side effects after prolonged opioid use. In the acute thermal pain hot-plate assay, the alkaloid extract (20 mg/kg) was found to produce a similar peripheral analgesic effect with greater efficacy than morphine. This analgesic effect may result from MOR activation, followed by intracellular cAMP reduction, and increased serotonin transmission. Chronic treatment with alkaloid extracts at doses of 8—45 mg/kg resulted less severe withdrawal symptoms than morphine, which was also observed in crude extract-treated mice. The withdrawal symptoms in morphine-treated mice are appear due to MOR activation and an increase in dopamine and serotonin level. Herein, morphine-dependent mice treated with alkaloid extracts showed increased serotonin levels while lowering dopamine. Moreover, in silico analysis suggests that the alkaloids compounds contained in the kratom extracts exhibit good binding activity to the TPH enzyme, which is likely related to increased serotonin release. Among the indole alkaloids, speciocialitine exhibited the highest binding affinity to TPH. These results indicate that serotonin is not only responsible for the analgesic effects, but also mediates the development of withdrawal symptoms in the chronic kratom extracts treatment.

Supplementary Information

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Supplementary Material 1.

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Authors' contributions
All authors played significant roles in the research and manuscript preparation processes. D.W.I. contributed to the conceptualization, methodology, data analysis, investigation, writing (original draft), visualization, review, and editing. S.I.R. contributed to the conceptualization, methodology, data analysis, writing (original draft), review, and editing. A.B. contributed to the data analysis, investigation, review, visualization, and editing. P.A. contributed to the investigation, review, and editing of the manuscript. A.N.S. contributed to the in silico study, data analysis, visualization, and editing. Z.Z. contributed to the writing clearance ethics for the study and investigation. N.L.P.I.D. contributed to the writing, review editing, and funding acquisition. M.Y.P. contributed to the supervision, writing, review, and editing.

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Data availability
All datasets used and/or analysed during the current study are available in the article and supplementary materials.

Declarations

Ethics approval and consent to participate
All experimental procedures, including the treatment of animals, were reviewed and approved by the Ethical Committee on Health of the National Research and Innovation Agency, Republic of Indonesia (053/KE.03/SK/05/2023).

Consent for publication
Not applicable.

Competing interest
The authors declare no competing interests.

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