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Erythroxylum Cuneatum Enhanced Receptor Internalisation on Morphine-Desensitised Neuroblastoma Cells Line

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Abstract

Chronic morphine exposure disrupts receptor signalling by inhibiting receptor internalisation, leading to receptor desensitisation and reduced availability of active receptors. This process significantly contributes to morphine addiction, a condition with limited effective and accessible treatments. Current pharmacological interventions, such as methadone, face challenges including restricted availability, risk of misuse, and adverse effects, leaving a substantial treatment gap for individuals with opioid use disorder (OUD). To address this, alkaloid extract of *Erythroxylum cuneatum* (*E. cuneatum*) was investigated as a potential alternative treatment. Using the human neuroblastoma cell line (SK-N-SH), 2 experimental designs were employed: Antagonistic treatment and pre-treatment with morphine. The antagonistic design evaluated the direct interaction between morphine and *E. cuneatum*, while the pre-treatment model assessed its anti-addictive properties during morphine withdrawal. Methadone served as a positive control. Cytosolic protein fractions were analysed to determine the expression of G protein-coupled receptor kinase 2 (GRK2), β-arrestin 1/2, and clathrin heavy chain, essential for receptor internalisation and resensitisation. Morphine exposure significantly downregulated these proteins, indicating impaired receptor function. Treatment with the alkaloid extract of *E. cuneatum* reversed these effects, restoring protein levels to those comparable with methadone. These findings highlight the alkaloid extract of *E. cuneatum* as a promising candidate for mitigating morphine addiction by targeting the molecular mechanisms underlying receptor desensitisation and resensitisation. *E. cuneatum* addresses a critical gap in addiction management by providing a potential alternative therapy that overcomes some limitations of existing treatments.

Keywords: *Erythroxylum cuneatum*, G protein-coupled receptor kinase 2 (GRK 2), β-arrestin 1/2, Clathrin heavy chain, Chronic morphine, Methadone, Addiction, Morphine desensitisation, Receptor internalisation

Introduction

Opiate dependence, withdrawal and relapses are significant contributors to societal burdens [1]. The introduction of opioid drugs initiates homeostatic processes explaining the natural adaptations for opioid addiction that reflect the induction phase of dependency [2]. Normal homeostasis develops opioid dependence triggered by exposure to the opioid drug. The biological basis of the cellular and synaptic adaptation of addiction to opioids is explained as tolerance, withdrawal symptoms, and the uncontrollable use of the drugs [3]. Chronic exposure to opiates, for example, morphine, causes the progress of plasticity in the brain, expressed by dependence and addictive symptoms.

Reactions of opioid drugs are claimed to be mediated by different types of receptors [4]. Morphine, for example, binds to 3 crucial classes of opioid receptors: μ-, κ-, and δ-opioid receptors [5-7]. Overactive receptors by morphine are observed in morphine dependence effects. The binding of morphine to the respective receptors activates the G-protein consisting of Gα- and βγ-subunits. Activation of different sub-units of G-protein; Gαi/Gαo, Gαq, and

Gαs, activates secondary messenger, which in turn expresses different reactions [8].

Chronic administration of morphine has been demonstrated to induce receptor desensitisation [9]. Morphine sensitisation alters the targeted neuron's neurotransmission and structure. The reaction induced by morphine is postulated to involve the G-protein coupled MORs. The receptor-activated by morphine, however, does not cause receptor internalisation, thus preventing the receptor desensitisation from occurring. The receptor internalisation is part of post-synaptic receptor trafficking [10]. This homeostatic mechanism happens in the neuron to ensure the synthesis of the receptor occurs and simultaneously sustain the concentration of active receptors. The persistent uncoupled G-protein and prolonged desensitisation due to chronic morphine cause addiction properties, including tolerance and dependence [9].

In regards to study the receptor desensitisation, resensitisation and internalisation, G protein-coupled receptor kinases 2 (GRK 2), β-arrestin 1/2, and clathrin heavy chain had been studies tremendously. These proteins play pivotal roles in regulating the endocytosis pathways critical to opioid receptor function and addiction mechanisms [9]. GRK 2 mediates the phosphorylation of G-protein-coupled receptors (GPCRs), including MOR, which facilitates the recruitment of β-arrestin 1/2 to the receptor [11]. This β-arrestin-mediated interaction triggers receptor desensitisation and internalisation, essential steps in terminating receptor signaling and initiating receptor recycling or degradation. Meanwhile, clathrin heavy chain forms clathrin-coated pits that are required for endocytosis, enabling the internalised receptors to traffic to endosomal compartments for further processing [12].

Dysfunction in this pathway, as observed in chronic opioid use, disrupts receptor desensitisation and recycling, leading to receptor hypersensitivity, tolerance, and dependency [13]. Chronic morphine exposure alters the activity of GRK 2, β-arrestin 1/2, and clathrin heavy chain, which are critical in the addiction mechanism. Chronic morphine administration suppresses the expression of GRK 2 and β-arrestin 1/2, impairing this critical desensitisation mechanism [14,15]. The resulting receptor hypersensitivity and inadequate recycling or degradation contribute to sustained MOR activation, which drives tolerance, dependency, and addiction behaviors. The activity of clathrin heavy chain in enabling receptor endocytosis also becomes compromised, exacerbating receptor dysregulation [16]. The ability to restore the function of GRK 2, β-arrestin 1/2, and clathrin heavy chain through therapeutic interventions is, therefore, critical to re-establishing receptor homeostasis and countering the maladaptive signaling associated with opioid addiction.

Erythroxylum cuneatum is a tropical plant found in substages of the primary and secondary rain and coral forests, distributed wildly in Southeast Asia, including Peninsular Malaysia [17]. Lv *et al.* [18] reported that the principal alkaloid of *E. cuneatum* was tropane alkaloid, one of the oldest organic compounds that possesses ethnopharmacological properties. Malaysia's *E. cuneatum* has not been fully explored but was claimed to have antioxidant and anti-inflammatory properties [19]. Interestingly, a study showed that the alkaloid extract of *E. cuneatum* possesses anti-addiction activity observed in morphine-addicted cell culture [20]. Based on the previous reports, the present study was designed to evaluate the probable effects of *E. cuneatum* on receptor sensitisation and internalisation in morphine-treated cells. The study used methadone, as an established anti-addiction drug, as a positive control to compare its effects to *E. cuneatum*.

Materials and methods

Materials

Human neuroblastoma cells line SK-N-SH (ATCC® HTB-11™) were purchased from American Type Culture Collection (ATCC, USA). Minimum Essential Medium (MEM), fetal bovine serum (FBS), penicillin-streptomycin were obtained from Gibco Life Technologies (Invitrogen, USA). Retinoic acid (RA) were purchased from Sigma-Aldrich (USA). Morphine sulphate pentahydrate (M-35-SU) and D,I-Methadone.HCl (MET-637) were purchased from Lipomed AG (Switzerland). Isobutylmethylxanthine (IBMX) and Radioimmunoprecipitation assay (RIPA) buffer, protease inhibitor and phosphatase inhibitor were purchased from Amresco, USA. Polyvinylidene difluoride (PVDF) membrane, 30 % acrylamide and bis-acrylamide solution (19:1) were purchased from Biorad, USA. The antibodies used, G protein-coupled receptor kinases 2 (GRK 2), β-arrestin 1/2, clathrin heavy chain,

anti-β-actin, and horse-radish peroxidase (HRP) were purchased from Cell Signalling Technology (Massachusetts). WesternBrightTM ECL and WesternBrightTM Peroxide were purchased from Advansta (USA).

Plant extraction

E. cuneatum leaves were collected from Bukit Broga, Selangor, Malaysia, and were deposited in the herbarium at Universiti Putra Malaysia (UPM) for species verification and identification (SK2100/12). The method of the alkaloid extract was optimised by the Forest Research Institute of Malaysia (FRIM) [20].

Cell culture

The human neuroblastoma cells line, SK-N-SH (ATCC® HTB-11™), was cultured in complete MEM containing Earle's salt and L-glutamine without sodium bicarbonate supplemented with 10 % of FBS and 1 % of penicillin/streptomycin. The neuroblastoma cells were cultured as a monolayer in an incubator at 37 °C in a humidified atmosphere of 5 % CO_2. The cell maintenance procedure was carried out following the manufacturer's prescription.

Retinoic acid (RA) on cell

The SK-N-SH cell line (2×10^5 cells), within passages 5 to 19, was seeded in a 24-well plate for 24 h at 37 °C with a 5 % CO_2 supply. The cells were introduced with 10 µM of retinoic acid (RA) (≥ 98 %, HPLC, Sigma) in 0.5 % DMSO for 6 days, alternately, to induce partial neuronal differentiation instead of massive proliferation, a characteristic of cancerous cells [20].

Morphine exposure

The experiment was designed with 2 different objectives: to observe the antagonistic activity of the extract and its effect on the morphine-induced cell line.

Antagonistic properties of morphine, *E. cuneatum* and methadone on receptor

The cells were co-treated with 50 µM of morphine sulphate pentahydrate and 50 µM of d,I-Methadone.HCl or 50 µM of morphine sulphate pentahydrate and alkaloid extract of *E. cuneatum* (0.1, 0.5, or 1.0 µg/mL) for 24 h.

Desensitisation or internalisation effect of morphine, *E. cuneatum* and methadone.

The cells were introduced to 50 µM of morphine sulphate pentahydrate for 24 h [21]. Then, the cells were washed with PBS before being treated with 50 µM of d,I-Methadone.HCl or alkaloid extract of *E. cuneatum* (0.1, 0.5, or 1.0 µg/mL) for subsequent 24 h. As for negative control, the cells were treated with 0.5 % DMSO in MEM. All drugs and alkaloid extract of *E. cuneatum* were prepared freshly by dissolving in 0.5 % DMSO.

Expression of biomarkers

The cells were harvested and lysed after the treatments. Protein concentration was determined using Bradford's reagent (Sigma Aldrich, USA). The aliquots of the cell lysates containing 20 µg of the proteins were separated using 10 % SDS-polyacrylamide gel and transferred to the PVDF membrane. The membrane was incubated with 5 % of skimmed milk for 2 h and incubated with anti-β-arrestin 1/2 (1:2500), anti-GRK 2 (1:2500), and anti-clathrin heavy chain (1:1000) at 4 °C overnight. Then, the membrane was washed and incubated with secondary antibody HRP (1:5000) for 2 h at 4 °C. The membrane was washed thrice with TBST for 10 min each, before being coated using chemiluminescent HRP Substrate, 1:1 of WesternBrightTM ECL and WesternBrightTM Peroxide. The membrane was visualised using Gel Documentation (GBOX-CHEM-HRI-4, UK) and the image of the protein of interest was obtained. Equal loading of samples was confirmed by β-actin antibody (1:1000). The intensity of the bands was measured using ImageJ [20].

Results and discussion

The antagonistic reaction of morphine, methadone and *E. cuneatum*

In the antagonistic treatment, GRK 2, β-arrestin 1/2 and clathrin heavy chain expressions were studied to observe the effect of the treatments on the receptor internalisation activity [22]. GRK 2 (**Figure 1**), β-arrestin 1/2 (**Figure 2**) and clathrin heavy chain (**Figure 3**) expressions significantly downregulated in the morphine-treated group ($p < 0.05$) compared to the control group, indicating suppression of the proteins.

Co-treatment of methadone with morphine shows a comparable expression of the protein to the control. Similarly, co-treatment of *E. cuneatum* with morphine demonstrated upregulation of GRK 2, β-arrestin 1/2 and clathrin heavy chain expressions expression as compared to the morphine treatment. The expression of dose-dependent *E. cuneatum* was comparable to control and methadone treatments.

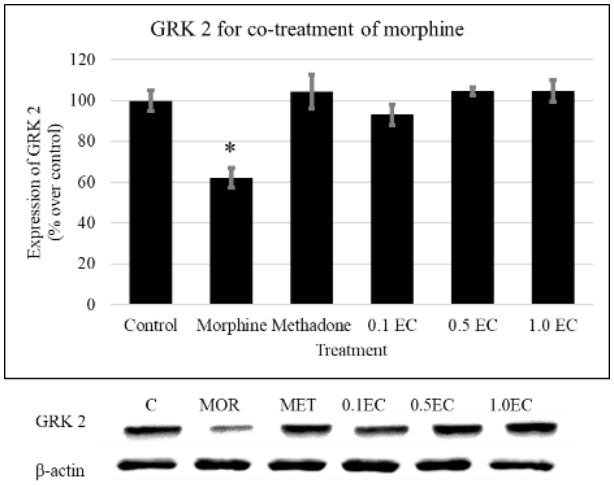

Figure 1 Expression of GRK 2 for antagonistic treatments of morphine, *E. cuneatum* and methadone. The bar chart shows that the expression of GRK 2 was downregulated in the morphine group ($p < 0.05$*) compared to the control, methadone and *E. cuneatum* (*$p < 0.05$). The expression of GRK 2 was visualised by the protein bands normalised against β-actin. (EC = Alkaloid extract of *E. cuneatum*; 0.1 = 0.1 µg/mL; 0.5 = 0.5 µg/mL; 1.0 = 1.0 µg/mL; C = Control; MOR = Morphine; MET = Methadone).

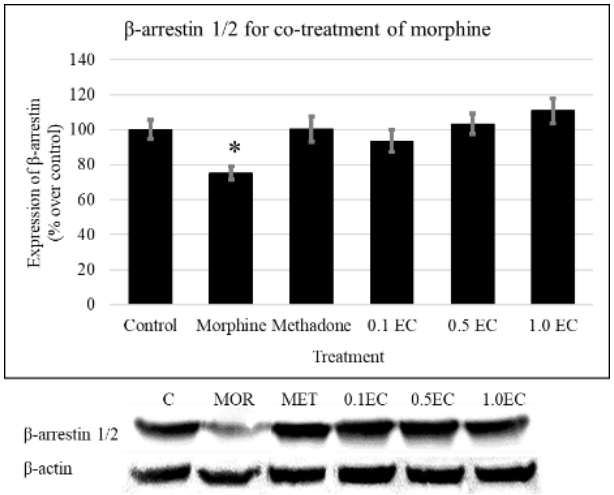

Figure 2 Expression of β-arrestin 1/2 for antagonistic treatments of morphine, *E. cuneatum* and methadone. The bar chart shows that the expression of β-arrestin 1/2 was downregulated in the morphine group ($p < 0.05$*) compared to the control, methadone and *E. cuneatum* (*$p < 0.05$). The expression of β-arrestin 1/2 was visualised by the protein bands normalised against β-actin. (EC = Alkaloid extract of *E. cuneatum*; 0.1 = 0.1 µg/mL; 0.5 = 0.5 µg/mL; 1.0 = 1.0 µg/mL; C = Control; MOR = Morphine; MET = Methadone).

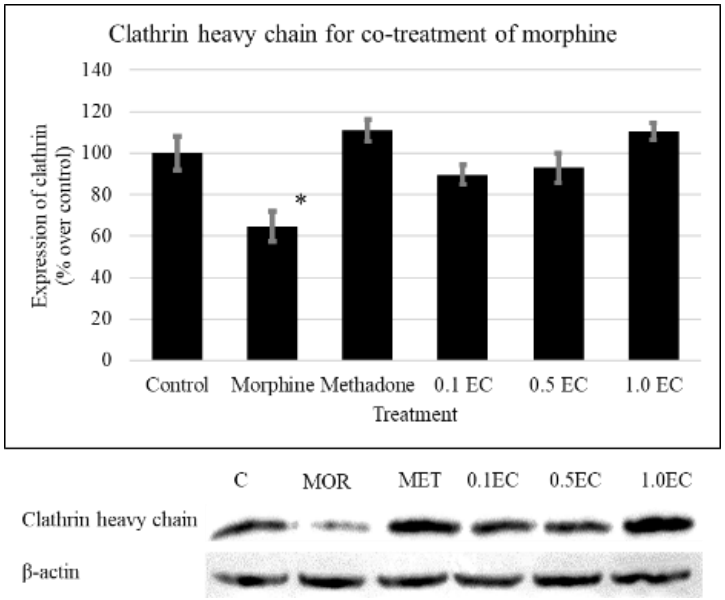

Figure 3 Expression of clathrin heavy chain for antagonistic treatments of morphine, *E. cuneatum* and methadone. The bar chart shows that the expression of clathrin heavy chain was downregulated in the morphine group ($p < 0.05$*) compared to the control, methadone and *E. cuneatum* (*$p < 0.05$). The expression of clathrin heavy chain was visualised by the protein bands normalised against β-actin. (EC = Alkaloid extract of *E. cuneatum*; 0.1 = 0.1 µg/mL; 0.5 = 0.5 µg/mL; 1.0 = 1.0 µg/mL; C = Control; MOR = Morphine; MET = Methadone).

The findings from this study provide critical insights into the potential antagonistic effects of *E. cuneatum* and methadone on morphine-induced downregulation GRK 2, β-arrestin 1/2 and clathrin heavy chain expression, important keys in receptor resensitisation in addiction mechanisms [11]. Prolonged morphine use is known to suppress GRK 2 expression, disrupting the phosphorylation and desensitisation of G-protein-coupled receptors (GPCRs), particularly MOR. This disruption leads to receptor hypersensitivity and contributes to the development of tolerance and dependence [23]. The upregulation of GRK 2 expression by methadone and *E. cuneatum* alkaloid extract suggests their role in mitigating the dysregulation of receptor signalling caused by morphine. Methadone, as a well-established opioid replacement therapy, restores GRK 2 expression [24] by engaging MOR by promoting receptor desensitisation and thereby reducing addiction presentation [25]. Similarly, the ability of *E. cuneatum* to normalise GRK 2 expression suggests its potential as a novel agent for receptor resensitisation, aligning with the therapeutic goals in addiction management.

In this study, morphine treatment resulted in a marked downregulation of β-arrestin 1/2, which reflects the impairment of receptor resensitisation, a process crucial for restoring receptor sensitivity after prolonged agonist exposure [11]. This disruption in receptor regulation is central to the development of tolerance and dependence in addiction. As β-arrestin 1/2 is involved in the internalisation and desensitisation of the MOR [26], its downregulation contributes to the persistence of MOR activation, thereby perpetuating addictive behaviours [27]. Interestingly, both methadone and *E. cuneatum* alkaloid extract demonstrated a normalisation effect on β-arrestin 1/2 expression, suggesting their potential to counteract morphine-induced receptor dysregulation. Methadone and *E. cuneatum* were shown to normalise the β-arrestin 1/2 expression comparable to the control group. This suggests their role in stabilising receptor function and reducing withdrawal symptoms [28]. Due to comparable findings to methadone, *E. cuneatum* is suggested to facilitate receptor resensitisation by modulating β-arrestin 1/2 expression and, consequently, reducing the dysregulation caused by morphine. These findings point to *E. cuneatum* as a promising candidate for anti-addiction therapies, providing a natural alternative or adjunct to traditional treatments like methadone.

Clathrin heavy chain is a key protein in receptor internalisation and recycling processes [29]. Its downregulation in the chronic morphine group disrupts the clathrin-mediated endocytosis of GPCRs, which is critical for receptor desensitisation and resensitisation. Impaired internalisation prevents proper receptor trafficking, leading to prolonged receptor activation and the persistence of addiction-related signalling pathways [11]. These findings align with previous studies indicating that chronic opioid exposure interferes with receptor recycling, contributing to the development of tolerance, dependence, and addiction. Interestingly, methadone and *E. cuneatum* alkaloid extract normalised the clathrin heavy chain expression comparable to the control group. Methadone, a widely used opioid substitution therapy, is known to stabilise receptor dynamics and mitigate addiction-related disruptions in receptor recycling. Comparable findings of methadone suggest that *E. cuneatum* exerts its effects by modulation of clathrin-mediated endocytosis, promoting receptor resensitisation and reducing the prolonged activation of morphine-induced MOR. The ability of *E. cuneatum* to normalise clathrin heavy chain expression underscores its potential as a natural anti-addiction agent, providing a promising alternative or adjunct to conventional therapies.

Desensitisation or internalisation effect of morphine, methadone and *E. cuneatum*

The mechanism of receptor internalisation after abrupt termination of chronic morphine was studied by the expressions of GRK 2, β-arrestin 1/2, and clathrin heavy chain [22]. GRK 2, β-arrestin 1/2, and clathrin heavy chain expressions were significantly downregulated in the morphine-treated group ($p < 0.05$) compared to other groups, as shown in **Figures 4** to **6**, respectively. In contrast, pre-treatment with *E. cuneatum* and methadone effectively normalised protein expressions comparable to control levels, demonstrating its ability to stabilise receptor desensitisation and mitigate the adverse effects of morphine on receptor regulation. These findings suggest that *E. cuneatum* promotes receptor internalisation and desensitisation by enhancing GRK 2, β-arrestin 1/2, and clathrin heavy chain expressions, thereby counteracting morphine-induced dysregulation.

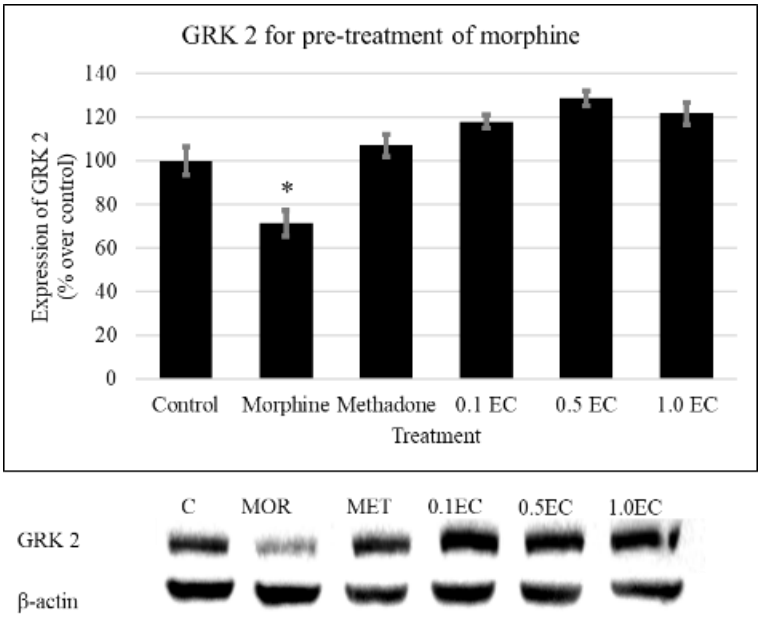

Figure 4 Expression of GRK 2 for pre-treatments of morphine, *E. cuneatum* and methadone. The bar chart shows that the expression of GRK 2 was downregulated in the morphine group ($p < 0.05$*) compared to the control, methadone and *E. cuneatum* (*$p < 0.05$). The expression of GRK 2 was visualised by the protein bands normalised against β-actin. (EC = Alkaloid extract of *E. cuneatum*; 0.1 = 0.1 μg/mL; 0.5 = 0.5 μg/mL; 1.0 = 1.0 μg/mL; C = Control; MOR= Morphine; MET= Methadone).

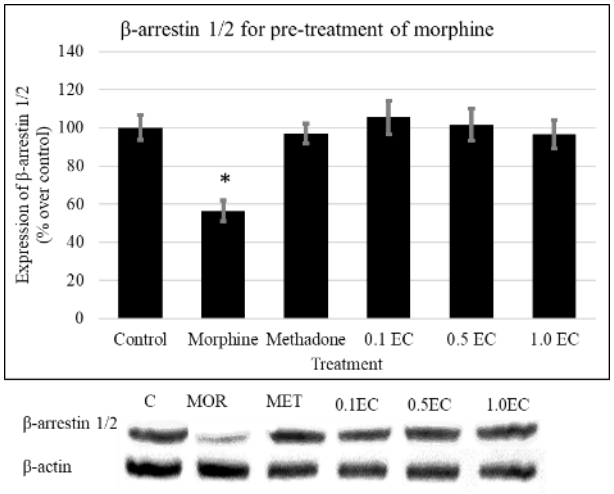

Figure 5 Expression of β-arrestin 1/2 for pre-treatments of morphine, *E. cuneatum* and methadone. The bar chart shows that the expression of β-arrestin 1/2 was downregulated in the morphine group ($p < 0.05$*) compared to the control, methadone and *E. cuneatum* (*$p < 0.05$). The expression of β-arrestin 1/2 was visualised by the protein bands normalised against β-actin. (EC = Alkaloid extract of *E. cuneatum*; 0.1 = 0.1 µg/mL; 0.5 = 0.5 µg/mL; 1.0 = 1.0 µg/mL; C = Control; MOR = Morphine; MET = Methadone).

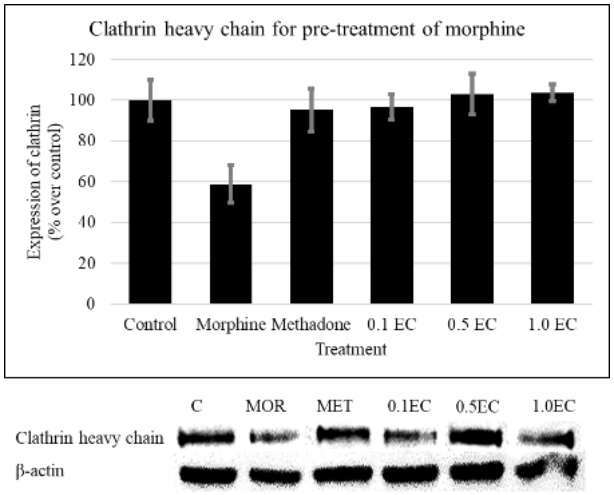

Figure 6 Expression of clathrin heavy chain for pre-treatments of morphine, *E. cuneatum* and methadone. The bar chart shows that the expression of clathrin heavy chain was downregulated in the morphine group ($p < 0.05$*) compared to the control, methadone and *E. cuneatum* (*$p < 0.05$). The expression of clathrin heavy chain was visualised by the protein bands normalised against β-actin. (EC = Alkaloid extract of *E. cuneatum*; 0.1 = 0.1 µg/mL; 0.5 = 0.5 µg/mL; 1.0 = 1.0 µg/mL; C = Control; MOR = Morphine; MET= Methadone).

GRK 2, β-arrestin 1/2, and clathrin heavy chain play a central role in regulating GPCR activity, particularly in receptor desensitisation, internalisation, and desensitisation [11]. In opioid addiction, GRK 2 phosphorylates activated MOR, facilitating their internalisation and subsequent recycling to restore receptor sensitivity [14]. Chronic morphine administration significantly downregulated GRK 2 expressions, which disrupts this critical regulatory pathway. Impaired receptor internalisation leads to prolonged receptor activation [30], contributing to tolerance, dependence, and the persistence of addictive behaviours [24]. Methadone and *E. cuneatum* normalised the GRK 2 expression comparable to control levels, indicating its role in stabilising receptor dynamics and mitigating morphine-induced receptor

dysregulation. Comparable findings suggest that *E. cuneatum* enhances receptor desensitisation and internalisation processes by regulating GRK 2 expression, thereby regulating MOR function.

The expression levels of β-arrestin 1/2 were assessed to evaluate their role in the desensitisation and internalisation of MOR [31] following pre-treatment with morphine, methadone, and *E. cuneatum* alkaloid extract. Abrupt termination of chronic morphine showed downregulation of β-arrestin 1/2 expression compared to other groups. This reduction indicates an impairment in receptor desensitisation and internalisation, processes critical for limiting receptor overstimulation and facilitating receptor recycling [15]. The downregulation of β-arrestin 1/2 highlights a key mechanism through which morphine disrupts normal receptor regulation, contributing to receptor overactivation, tolerance, and addiction. In contrast, treatment of methadone and *E. cuneatum* after the abrupt termination of chronic morphine normalised the β-arrestin 1/2 expression comparable to the control group. The findings suggest their role in stabilising receptor desensitisation and mitigating the adverse effects of morphine. *E. cuneatum* is postulated to promote receptor internalisation and regulate MOR signalling by modulation of β-arrestin 1/2 expression.

Clathrin heavy chain is a critical component of clathrin-coated vesicles responsible for receptor internalisation, a process essential for regulating and recycling GPCRs, including the MOR [12]. The reduced expression of the clathrin heavy chain observed in the abruptly terminated morphine group suggests impaired receptor internalisation, leading to prolonged receptor activation and exacerbation of addiction-related signalling [16]. The treatment of methadone and *E. cuneatum* after the abrupt termination of chronic morphine normalised the expression of clathrin heavy chain comparable to the control group. The findings demonstrate their role in stabilising receptor internalisation and recycling processes disrupted by chronic morphine. These results highlight the therapeutic potential of *E. cuneatum* in counteracting morphine-induced impairments in receptor internalisation by restoring clathrin heavy chain expression. This suggests that *E. cuneatum* promote receptor regulation and trafficking, contributing to the normalisation of receptor signalling disrupted in opioid addiction.

Desensitisation, internalisation and resensitisation of receptor

The bioactive compounds of *E. cuneatum*, particularly alkaloids, flavonoids, and phenolic acids, offer diverse health benefits. These compounds exhibit anti-inflammatory, antioxidant, and analgesic properties, making the plant valuable for general health [32]. The anti-inflammatory effects help manage chronic pain and reduce inflammation-related conditions, while the antioxidant properties combat oxidative stress, promoting overall cellular health [19]. Additionally, its bioactive compounds have potential neuroprotective effects, supporting brain health and potentially aiding stress management and neurological disorders. *E. cuneatum* holds promise as a natural therapeutic agent for improving overall well-being [33].

Alkaloid compounds of *E. cuneatum* are known for their anti-inflammatory, analgesic, and neuroprotective properties [32], suggesting their potential role in managing conditions associated with chronic pain and opioid addiction [20]. The alkaloids in *E. cuneatum* are thought to modulate key cellular signalling pathways, including those involving GPCRs and β-arrestin-mediated signalling, which are critical in receptor desensitisation and resensitisation processes. The multifaceted pharmacological actions of these compounds underscore the potential of *E. cuneatum* as a natural source for developing anti-addiction therapies and adjunct treatments for opioid dependency.

In the present study, the desensitisation or internalisation of the receptor was analysed by the expression of GRK 2, β-arrestin 1/2 and clathrin heavy chain. Low expression of GRK 2 and β-arrestin 1/2 suggests receptor desensitisation [11]. Li *et al.* [17] postulated that the recruitment of GRK 2 and β-arrestin 1/2 of low-efficacy opioids, such as morphine, promotes MOR desensitisation. The desensitisation of MOR is proclaimed to induce addiction [13]. In contrast, the overexpression of GRK 2 and β-arrestin 1/2 are related to the increase of the sequestration process [11]. Sequestration is crucial in receptor internalisation to regulate the activated receptor into the intracellular membrane (endocytosis process) and intracellular trafficking [34]. Following the endocytosis process by

the β-arrestin 1/2, the process is induced by the clathrin heavy chain, which acts as a vesicle to direct the endocytic machinery component, consisting of the complex of β-arrestin 1/2 and clathrin [11]. The complex is responsible for receptor internalisation and resensitisation (**Figures 7** and **8**). The activated receptor stimulates the β-arrestin 1/2 to trigger the plasma membrane in forming a doughnut shape. The complex of β-arrestin 1/2 activates the clathrin-coated vesicle to start the endocytosis process.

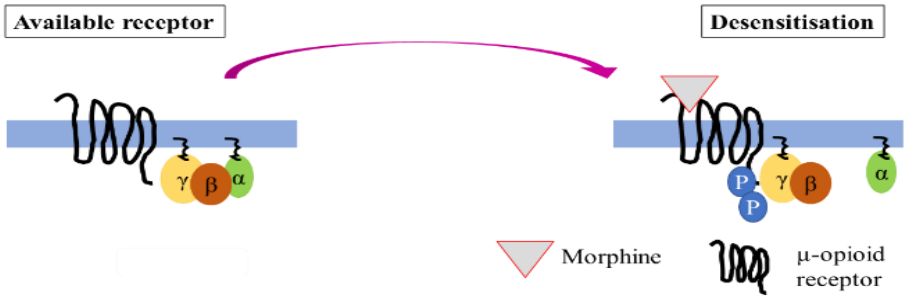

Figure 7 Summary of receptor desensitisation after chronic morphine treatment. Chronic morphine treatment leads to receptor desensitisation, predominantly at the MOR. This process typically involves phosphorylation of MOR by GRK 2, recruitment of β-arrestin 1/2, and subsequent endocytosis mediated by clathrin heavy chain. However, chronic morphine exposure downregulates the expression of GRK 2, β-arrestin 1/2, and clathrin heavy chain, impairing the mechanisms of receptor internalisation and desensitisation. The reduced internalisation prevents receptor trafficking to intracellular compartments, where they are dephosphorylated and recycled back to the membrane, resulting in prolonged receptor desensitisation and contributing to tolerance and addiction [13].

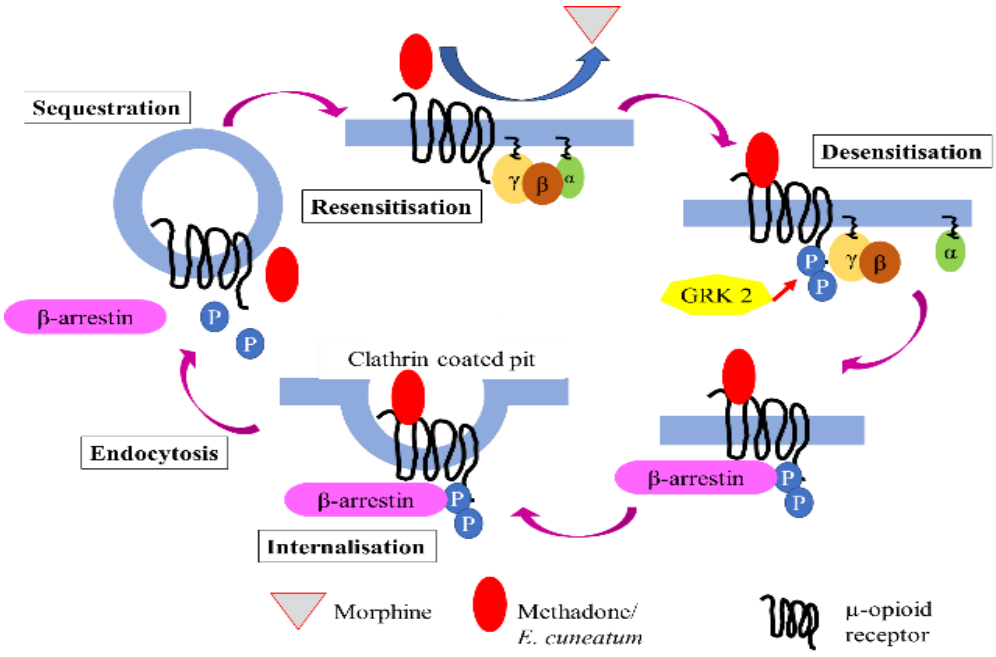

Figure 8 Summary of receptor desensatisation, endocytosis, internalisation, sequestration, and resensitisation promoted by methadone and *E. cunaetum*.

This figure illustrates the receptor regulatory processes, including desensitisation, endocytosis, internalisation, sequestration, and resensitisation. Chronic morphine use leads to receptor desensitisation, initiated by (GRK 2-mediated phosphorylation of the receptor, which facilitates the binding of β-arrestin 1/2. β-arrestin 1/2 prevents further receptor signalling and recruits clathrin heavy chain, promoting receptor

endocytosis and internalisation into endosomes. Sequestration of internalised receptors allows for dephosphorylation and recycling of receptors to the plasma membrane, restoring their functional sensitivity-receptor resensitisation. Co-treatments and pre-treatment with methadone and *E. cuneatum* enhance these processes by restoring GRK 2, β-arrestin 1/2 expression and clathrin heavy chain, promoting balanced receptor regulation. Methadone acts as a partial agonist at the MOR, facilitating less disruptive receptor modulation, while *E. cuneatum*'s bioactive compounds potentially stabilise GPCR regulation, offering a novel anti-addiction mechanism [11].

The activated receptor by morphine underwent desensitisation without subsequent endocytosis and internalisation of the receptor [9]. This study has shown that the combination of morphine with *E. cuneatum* normalised the level of the proteins, suggesting that *E. cuneatum* induces receptor internalisation and resensitisation despite the presence of morphine. The data was comparable to the combination treatment of morphine and methadone. The morphine was known to have a low rate of endocytosis of the desensitised MOR, thus expressing the morphine withdrawal symptom [13]. In contrast, comparable findings with methadone postulate the role of *E. cuneatum* in treating withdrawn cells by normalising the level of GRK 2, β-arrestin 1/2 and clathrin heavy chain.

Conclusions

The findings of this study provide compelling evidence for the potential of *E. cuneatum* alkaloid extract as an antagonist to morphine-induced receptor desensitisation and dysregulation in neuroblastoma cell lines. Chronic morphine exposure resulted in significant downregulation of GRK 2, β-arrestin 1/2, and clathrin heavy chain, key proteins essential for receptor desensitisation, internalisation, and resensitisation processes. Co-treatment with *E. cuneatum* normalised the expression of these proteins, demonstrating effects comparable to those of methadone. These findings suggest that *E. cuneatum* promotes receptor internalisation and desensitisation, mitigating morphine-induced dysregulation and providing a promising plant-based alternative for anti-addiction therapy. The observed normalisation of receptor regulatory proteins highlights the potential role of *E. cuneatum* in stabilising MOR signalling and reducing addictive behaviours. *E. cuneatum*, a non-opioid alternative, could provide safer and broader therapeutic benefits, including potential anti-inflammatory effects that may address comorbid conditions in addiction. These multifaceted pharmacological actions of *E. cuneatum* underscore its potential as a novel candidate for anti-addiction treatments targeting receptor dynamics disrupted by chronic opioid use.

Despite these promising findings, this study is limited in evaluating the effects of *E. cuneatum* on receptor internalisation and desensitisation mechanisms in a morphine-desensitised neuroblastoma cell model. The anti-addiction effects of *E. cuneatum*, including its impact on withdrawal symptoms, tolerance, and dependence, remain to be fully elucidated. Future studies should explore the anti-addiction properties of *E. cuneatum* using *in vivo* models of opioid addiction to validate its efficacy and safety over longer treatment durations. Additionally, the active alkaloid compounds within *E. cuneatum* should be characterised to identify their specific molecular targets and pathways. Investigating the interactions of EC with addiction-related signalling cascades, such as the NF-κB and MAPK pathways, will provide further insights into its therapeutic potential. These efforts could pave the way for the development of EC as a novel, plant-based treatment for opioid addiction.

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