Original Research Article (Experimental)

Pomegranate-derived anthocyanin regulates MORs-cAMP/CREB-BDNF pathways in opioid-dependent models and improves cognitive impairments

Norhaslinda Ridzwan a, Mimie Noratiqah Jumlia a, Atif Amin Baig b, Mohd Adzim Khalili Rohin a, *

a School of Nutrition and Dietetic, Faculty of Health Sciences, Universiti Sultan Zainal Abidin (UniSZA), Gong Badak Campus, 21300, Kuala Nerus, Terengganu, Malaysia

b Faculty of Medicine, Universiti Sultan Zainal Abidin (UniSZA), Medical Campus, 20400, Kuala Terengganu, Terengganu, Malaysia

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A B S T R A C T

Background: Pomegranate (Punica granatum) is one of the oldest known edible fruit. Recently, there has been an increased interest in this fruit as a functional food for health benefits due to its use in disease prevention and promotion of overall health wellness.

Objective: This study aims to investigate the effects of pomegranate extract for the development of non-opioid substitution therapy for in-vitro and in-vivo studies.

Materials and methods: Anthocyanin contents consisting of cyanidin 3-glucoside, diglucoside, and pelargonidin 3-glucoside, diglucoside were detected and quantified in pomegranate extract using high-performance liquid chromatography. The optimum dosage of the extract was determined based on the regulation of MORs and cAMP proteins in U-87 cells. Co-treatment of the extract with morphine was performed to evaluate its potency in reducing the concentration levels of MORs and cAMP. For animal studies, rats were divided into two major groups representing both acute and chronic morphine-induced treatments and the Morris water maze (MWM) study was employed after treatment for each rat. The rats were sacrificed after the treatments and serum samples were collected to evaluate the levels of CREB and BDNF.

Results: The results indicated that each of the anthocyanin content tested in the study was present in the pomegranate extract. Additionally, in-vitro studies using pomegranate extract treatment showed that the extract was effective in decreasing the MORs and cAMP protein levels in U-87 cells at a concentration of 0.125 mg/mL. The memory impairment based on the MWM study in rats was also subsequently improved after treatment with pomegranate extract as compared to treatment with morphine. The blood serum derived from the rats treated with pomegranate extract also showed a significant decrease in CREB level and an increase in BDNF as compared to rats treated with morphine.

Conclusion: In conclusion, this study substantiates the potency of pomegranate extract as a non-opioid substitution therapy for in-vitro and in-vivo studies.

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1. Introduction

The term ‘opioids’ refers to all the natural and synthetic compounds that are functionally related to opium derived from the poppy plant, Papaver somniferum and endogenous neuropeptides [1]. Opioids are a class of drugs that are prescribed as pain relievers such as morphine, codeine, oxycodone, fentanyl, and heroin [2]. In the brain, the opioid receptors are divided into three major types known as \( \mu \) (Mu), \( \delta \) (Delta) and \( \kappa \) (Kappa) receptors [3]. The \( \mu \)-opioid receptors (MORs), derived from the Oprm1 gene, are the main molecular targets for opiate-mediated analgesia and belong to the G protein-coupled receptor (GPCR) superfamily that possesses a 7-trans-membrane-spanning domain architecture [4,5].

* Corresponding author.
E-mail: mohdadzim@unisza.edu.my

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To date, many researchers have been actively focusing on the neurobiological processes involved in addiction, tolerance, dependence, and withdrawal of opioids [4,6–8]. In addition, several addictions and addictive treatments of opioid-related disorders have also recently surfaced through the diverse pharmacological, psychological, social intervention approaches such as methadone maintenance therapy [9]. However, the duration of the treatment usually lasts from just a few days to years with declining success rates, in which the treatment depends on the individual needs and availability of resources [10]. Furthermore, it has been shown that the use of modern drugs also exerts various side effects on individuals with opioid dependence which include severe withdrawal effects which are either physical or/and mental [9].

There have been several studies exploring the use of non-opioid drugs from potential natural sources such as honey, Nigella sativa, and Phoenix dactylifera [11–13]. However, much of the attention has shifted towards the antioxidant properties found in fruits, namely due to the increasing epidemiological and pharmacological evidence of biologically active compounds that are thought to offer health benefits against various diseases [14]. Recently, there is an increased interest in pomegranate fruit as a functional food due to its abundant source of polyphenols when compared with other fruits against various diseases [14]. Recently, there is an increased interest in pomegranate fruit as a functional food due to its abundant source of polyphenols when compared with other polyphenol-rich beverages such as wine and green tea [15]. Additionally, pomegranate fruit also contains rich sources of bioactive compounds which include phenolic acids, tannins, flavonoids and anthocyanins [16], thereby indicating its efficacy as a therapeutic agent with health benefits for the treatment of atherosclerosis, cancer and neurodegenerative disorders [16,17]. Therefore, this study aims to investigate the effects of pomegranate (Punica granatum) extract using a series of in-vitro and in-vivo studies for the development of non-opioid substitution therapies.

2. Materials and methods

2.1. Collection of pomegranate fruits

Fresh pomegranate fruits were obtained from a market in Kuala Terengganu, Terengganu, Malaysia. The plant material was authenticated by the Institute of Bioscience, Universiti Putra Malaysia with a voucher serial number of SK 3360/16. The fruits were carefully washed under running tap water and dried with a soft cloth. The skin of the fruit was peeled and the fresh flesh (aril with intact seeds) was separated from the peel.

2.2. Extraction of pomegranate fruit

The method introduced by Mphahlele et al. [18] was used in this study with slight modifications. The fresh aril flesh with intact seeds was juiced using a Waring 2-speed blender (240 VAC-Standard Motor). The pomegranate juice was subjected to centrifugation at 1780 g for 5 min and pellets were discarded to remove any fibrous material. Approximately 50 g of flesh was soaked in water (1:10; w/v) for 24 h at room temperature (24–25 °C). The extracts were filtered using the Whatman® No. 41 filter paper with a pore size of 20–25 μm and concentrated using the rotary evaporator at a temperature of 40 °C. The concentrated extracts were then placed in the oven at 50 °C to allow for the complete evaporation of solvents. The extracts were stored in −80 °C prior to analysis.

2.3. Determination of anthocyanin content in pomegranate extracts using high-performance liquid chromatography (HPLC)

2.3.1. Preparation of anthocyanin standards

Approximately 1 mg of anthocyanin standards were prepared, in which cyanidin 3-glucoside, cyanidin 3, 5-diglucoside, pelargonidin 3-glucoside, and pelargonidin 3,5-diglucoside were dissolved in 1 mL of 100% methanol (HPLC grade) to obtain a stock solution of 1000 μg/mL. Each anthocyanin standard was diluted to obtain a working solution of six different concentrations at 5, 10, 15, 25, and 30 μg/mL which was used to make a standard curve. The stock solutions were stored in −80 °C prior to analysis.

2.3.2. Preparation of pomegranate extract

Approximately 10 mg of pomegranate extract was dissolved in 1 mL of 100% methanol (HPLC grade) to obtain a stock solution of 10 mg/mL. The stock solution was filtered through a 0.45 μm PTFE hydrophobic syringe filter (Bionflow, China) into a HPLC vial before injection into the HPLC system.

2.3.3. Determination of anthocyanin in pomegranate extract

The anthocyanin determination method introduced by Gomez-Caravaca et al. [19] was used in this study with slight modifications. Briefly, the characterization of anthocyanin was performed using HPLC equipped with a system gold programmable detector module 166-UV-Vis (Beckman Coulter, USA) and a LiChroCART 100 RP-18 column with an internal diameter size dimension of 25 cm × 0.4 cm and particle size of 5 μm (Merck, Germany). The mobile phase solvents consisted of 0.001% T-fluoro acetyl acid and deionized water (A), 100% methanol (HPLC grade) (B), and 100% acetonitrile (HPLC grade) (C). The 3-glucoside and 3, 5-diglucoside cyanidin standards were set using gradient 3 and ran for 20 min. The 3-glucoside and 3, 5-diglucoside pelargonidin standards were set using an isocratic gradient and ran for 10 min. The flow rate was set at 0.3 mL/min. The chromatograms were recorded at an absorbance of 260 nm. The standard curves of each anthocyanin standard were plotted based on five different concentrations of 5, 10, 15, 20, and 30 μg/mL. The anthocyanin content in pomegranate extracts was determined and quantified based on the chromatograms and retention times (RTs) of anthocyanin standards consisting of cyanidin 3-glucoside, cyanidin 3, 5-diglucoside, pelargonidin 3-glucoside, pelargonidin 3, 5-diglucoside (Sigma, Co. Chemical, St Louis, USA) which was used as a reference for quantification.

2.4. In-vitro studies

2.4.1. Preparation of pomegranate extract

A stock solution of pomegranate extract (10 mg/mL) was prepared by dissolving 10 mg of extract in 1 mL of DMSO. All extract solutions were kept at 4 °C throughout the analysis. Stock solutions were further diluted in RPMI-1640 (Gibco, Invitrogen, USA) media supplemented with 10% fetal bovine serum (Gibco, Invitrogen, USA) and 1% penicillin/streptomycin (Gibco, Invitrogen, USA), in which a final concentration of 1 mg/mL was obtained and used for further tests.

2.4.2. Cell maintenance and harvesting

A human glioblastoma multiforme cell line, U-87 MG (ATCC® HTB-15™), was used to express the MORs. The cell line was obtained at the 5th passage (PS) from the cell bank of the Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin
(UniSZA). The U-87 cell lines were harvested and maintained in an incubator at 37 °C with 95% relative humidity and 5% CO2 in RPMI-1640 (Gibco, Invitrogen, USA) media supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, USA) and 1% of penicillin/streptomycin (Gibco, Invitrogen, USA). According to Byrne et al. [20], the U-87 cell line has been shown to express all three types of opioid receptors. Additionally, the expressed MORs were confirmed to be functionally active in the U-87 cell line with 3.43 × 10^7 ± 0.67 × 10^7 copies of MORs/μg of total RNA. Hence, in this study, the U-87 cell line was used as an opioid-expressing cell to measure the effect of morphine sulfate and pomegranate extract on MORs and cAMP protein concentrations.

### 2.4.3. Determination of safe dosages for morphine sulfate and pomegranate extract

The microtitration colorimetric method based on the tetrazolium salt reduction assay, also known as the MTT assay, was used to evaluate the safe dosages for morphine sulfate and pomegranate extract. The specific range of safe dosages was crucial to be determined as it was required for further tests in this study. The MTT assay was performed based on the viability of cells which was assessed using the trypan blue method [21]. The cells were harvested, counted using the hemocytometer and diluted in a culture medium. An aliquot of 100 μL cell suspension was dispensed in triplicates into a 96-well culture plate (SPL Life Sciences, Korea) with an optimized density of 1 × 10^4 cells/cm². After a 24-h recovery period, a serial dilution was performed to obtain various concentrations of pomegranate extract (1.0, 0.5, 0.25, 0.125, 0.0625, 0.0313, and 0.0156 mg/ml) and morphine (10.0, 5.0, 2.5, 1.25, 0.625, 0.313, and 0.156 μg/ml) which were added into each well containing the cell suspensions in triplicates. After 72 h of incubation, 20 μL of MTT (Fisher Scientific, USA) assay was added into each well and re-incubated for 4 h. The culture media was subsequently removed and 100 μL of DMSO was added into each well to solubilize the formazan crystals. In the final step of the procedure, absorbance was measured at a wavelength of 570 nm using a microplate reader (Tecan, Infinite M200, Switzerland).

### 2.4.4. Cell harvesting and treatments

The U-87 cell line was used to measure the effect of morphine sulfate and pomegranate extract on MORs and cAMP protein concentrations. The optimum dosages of pomegranate extract and morphine were determined by treatment with final concentrations of pomegranate extract and morphine in U-87 cells for 3 and 48 h, respectively [20]. Subsequently, a time response study was conducted by incubating the U-87 cell lines with the selected optimum dosage concentration of morphine and pomegranate extract for 48 h [20]. For the negative control, cells were grown in culture media without any treatment. The treatment groups in this study consisted of the negative control, morphine, morphine and naloxone (100 μM) [1], morphine and methadone (6.25 μg/ml) [1], and morphine and pomegranate extract. The supernatants for each treatment and control group were subsequently harvested to measure the MORs and cAMP protein concentration levels.

### 2.4.5. Collection of cell lysates

The cell lysate samples were collected according to the protocol established for the human MORs (Cusabio Biotech, China) and cAMP (Cell Biolabs, Inc, San Diego, USA) ELISA kit assays. The treated and non-treated U-87 cells in petri dishes (Iwaki, Japan) were harvested using the cell scraper (SPL Life Sciences, Korea) and stored at −20 °C in 500 μL of PBS (Gibco, Invitrogen, USA) solution. After two cycles of freezing and thawing, the cell lysates were subjected to centrifugation (5000 × g) at 4 °C for 5 min. Cell lysates were carefully removed to avoid the pellet and were collected in a micro-centrifuge. The lysates were aliquoted and stored in a −20 °C freezer until required for further use to avoid multiple freeze/thaw cycles.

### 2.4.6. Quantification of MORs concentration level

The MORs concentration level was measured using the MORs ELISA kit assay purchased from Cusabio Biotech (China). This assay has high sensitivity and excellent specificity for the detection of human MORs within the range of 31.25–2000 pg/mL. No significant cross-reactivity or interference between the human MORs and analogs was observed using this assay. An aliquot of 100 μL of cell lysates was assayed directly in the antibody-coated wells for 2 h prior to the addition of 100 μL of biotin antibody and re-incubation for 1 h. Subsequently, the wells were washed and 100 μL of HRP-avidin was added to each well with a further re-incubation for 1 h. After the washing step, approximately 90 μL of TMB substrate was added and the plate assay was re-incubated for 15 min. The reaction was stopped by the addition of 50 μL of stop solution and the absorbance was measured at 540 nm using a microplate reader (Tecan, Infinite M200, Switzerland).

### 2.4.7. Quantification of cAMP concentration level

The cAMP concentration level was measured using the cAMP ELISA kit (Colorimetric) assay purchased from Cell Biolabs, Inc (San Diego, USA). Approximately 50 μL of cell lysates, 25 μL of diluted peroxidase cAMP tracer conjugate solution, and 50 μL of diluted polyclonal antibody were assayed directly in the antibody-coated wells for 2 h. Subsequently, the wells were washed and 100 μL of substrate solution was added and re-incubated for 5 min. The reaction was stopped by the addition of 100 μL of stop solution and the absorbance was measured at 450 nm using a microplate reader (Tecan, Infinite M200, Switzerland).

### 2.5. In-vivo studies

#### 2.5.1. Ethics approval

The ethics approval for the animals used in this study was obtained from the Animal & Plant Research Ethics Committee (UAPREC), Universiti Sultan Zainal Abidin (UniSZA) with an approval permit reference number of UAPREC/17/007.

#### 2.5.2. Study design

The rats were divided into 8 sub-groups consisting of four rats each for the acute and chronic morphine administration groups, in which four treatments consisting of the negative control (no-treatment), morphine, morphine treated with methadone, and morphine treated with pomegranate extract were performed. These subgroups were chosen to investigate the effects of pomegranate extract as a non-opioid substitution therapy in this study. The morphine treatment group acted as a positive control, while the use of methadone treatment acted as a comparison for the pomegranate extract treatment groups. The rats were subjected to an acclimatization period for 3 days prior to the experiments.

#### 2.5.3. Sample size

The sample size of animal was calculated by using a crude method based on law of diminishing return, in which known as resource equation method [22].
E = Total number of animals − Total number of groups

The value of E should be between 10 and 20 to be considered as an adequate and more than 20 is considered as more than adequate [22]. The total number of animals used in the present study is 32 and the total number of groups is 8 and the number of rats used per group is 4, therefore E is 24, which is more than adequate.

2.5.4. Experimental animals

In total, 32 male Sprague–Dawley rats weighing between 200 and 250 g were used in this study. The rats were identified as SD-M model with nomenclature NTac:SD. The rats were purchased from a supplier in Johor (Forensic Animal Supply, Johor, Malaysia) and maintained in the animal house facility at the Faculty of Medicine, Universiti Sultan Zainal Abidin (UniSZA), Terengganu, Malaysia. The Sprague–Dawley rats were kept in individual cages and subjected to a light–dark cycle with 12 h of light and 12 h of darkness. The rats were given free access to food and water during the experimental period.

2.5.5. Experimental procedures

2.5.5.1. Morphine administration in rats. For acute morphine administration, morphine was injected once with a dose of 15 mg/kg via the intraperitoneal (i.p.) route. For chronic morphine administration, morphine was i.p injected with an increasing daily dose from 6 mg/kg to 66 mg/kg, starting from day 2 until day 8 [23]. For acute and chronic morphine administration, methadone (0.2 mL/kg) [24] and pomegranate extract (600 mg/kg) [23] were administered orally by oral gavage for seven days after inducing with naloxone (0.5 mg/kg) (i.p.) [24]. For the negative control treatments, the acute and chronic dosage administrations were i.p injected with 1 mL/kg of saline.

2.5.5.2. Preparation of pomegranate extract dosage. A concentration of 600 mg/kg of pomegranate extract was given as a daily oral administration for the experimental groups treated with pomegranate extract based on the recommended dosage by Patel et al. [23]. It was proposed that this dosage did not cause any biologically significant adverse effects and was safe to be consumed based on the evaluations performed by biochemical analysis and histological examinations on the bodyweight changes in rats. The dosage was prepared according to the established OECD guidelines [24]. Briefly, approximately 60 mg of pomegranate extract was dissolved in 1 mL of normal saline to obtain a dosage concentration of 600 mg/kg/day for a rat with an approximate weight of 100 g. The stock solutions for 8 rats were further prepared, in which 480 mg of pomegranate extract was diluted in 8 mL of normal saline to obtain a final concentration of 60 mg/mL (from a selected dose of 600 mg/kg/day) which was subsequently used for treatment.

2.5.5.3. MWM study. The MWM model established by Vorhees and Williams [25] and Rezvani-Kamran et al. [26] was used in this study with some slight modifications to assess the memory impairment of morphine-dependent rats with withdrawal syndromes. After treatment, each rat was given a swim training consisting of four trial sessions for 60 s in the presence of a platform for 4 consecutive days. The rats spent 30 s on the platform between each trial and were allowed to rest for 15 min between the two consecutive trials. If the animal failed to locate the platform within 60 s, it was gently directed to the platform by the researcher and allowed to stay there for 30 s. The point of entry for the rat remained unchanged for trials 1, 2, 3, and 4 within 4 consecutive days. On day 5, each rat was given two swim trial sessions for 60 s in the absence of the platform. During each trial, the duration taken to swim and distance of rats swimming to the platform was recorded by a video camera (Nikon, Melville, NY) linked to a stand placed directly above the pool. This parameter was averaged for each trial session for each rat individually. The model in this study differed slightly to the previous study by Rezvani-Kamran et al. [26], in which two trial sessions in the absence and presence of the platform (covered with an aluminum foil) were performed. Additionally, the previous study allowed the rats to rest for only 5 min between the two consecutive trials compared to this study which employed a rest interval of 15 min. Apart from these modifications, the model applied in this study was performed accordingly to the method by Rezvani-Kamran et al. [26].

2.5.5.4. Preparation of animals for anaesthesia. Anaesthetic combination of TKX (Tiletamine, Ketamine and Xylazine) was applied to the rats as a sedative to induce anaesthesia condition. It was prepared according to the method applied by Williams et al. [27] with some slight modifications. A bottle of Zoletile (Tiletamine Hydrochloride and Zolazepam Hydrochloride) in powder form was reconstituted with 2.5 mL of Ketamin (100 mg/mL) and 12.5 mL of Xylazine (100 mg/mL). In order to prepare 30 mL of TKX anaesthetic, 2 mL of TKX combination was diluted with 8 mL of saline. The standard dose for an adult rat is 0.01 mL/100 mg was injected via intra-venous (i.v) at tail-vein. The dosage was adjusted according to the weight of the rats. Each of rat was anaesthetic once during the period of study.

2.5.5.5. Blood collection procedure. The anaesthetic (i.v) injection was given after treatment for blood drain via cardiac puncture. Blood was collected once during post treatment for each of rats. After treatment, 5 mL of blood was collected for each rats. Each of blood sample was centrifuged to get the serum; 1 mL blood contain about 55% of serum. All the blood was collected via cardiac puncture of blood collection procedure [28].

2.5.5.6. Quantification of CREB concentration level. The CREB concentration level was evaluated using a Phospho-CREB (Ser 133) sandwich ELISA kit (Cell Signalling Technology, USA). Approximately 100 μL of serum was added into the well and incubated for 2 h. The wells were washed and 100 μL of re-constituted detection antibody solution was added and re-incubated for 1 h. The wash procedure was repeated and 100 μL of TMB substrate was added and re-incubated for 10 min. The reactions were stopped by adding 100 μL of stop solution to each well and the absorbance was measured at 450 nm using a microplate reader (Tecan, Infinite M200, Switzerland).

2.5.5.7. Quantification of BDNF protein concentration level. The BDNF concentration level was evaluated using a BDNF ELISA assay (Abcam, United Kingdom). Approximately 100 μL of serum was added into a 96-well plate with antibody coating and incubated for 2.5 h. Subsequently, the wells were washed and 100 μL of biotinylated anti-human BDNF detector antibody solution was added and re-incubated for 1 h. After the washing step, 100 μL of HRP-streptavidin solution was added and re-incubated for 45 min. The solution was discarded and washed prior to the addition of 100 μL of TMB substrate reagent and re-incubation for 30 min. The reactions were stopped by adding 50 μL of stop solution and the absorbance was measured at 450 nm using a microplate reader (Tecan, Infinite M200, Switzerland).

2.6. Statistical analysis

The statistical data analysis was performed using the statistical package for the social sciences (SPSS) Version 20.0 software (IBM Corp. Armonk, NY, US). All values were expressed as the mean (SD)
3. Results

3.1. Determination of anthocyanin in pomegranate extract using HPLC

3.1.1. Validation of anthocyanin standards

Based on Table 1, the LOD and LOQ values of each anthocyanin standard were evaluated to determine the methodology sensitivity and suitability for analysis. In this study, the LOD values obtained were 3.32, 11.36, 11.32, and 5.40 μg/mL for cyanidin 3-glucoside, cyanidin 3, 5-diglucoside, pelargonidin 3-glucoside, and pelargonidin 3, 5-diglucoside, respectively. The LOQ values obtained were 10.05, 34.43, 34.29, and 16.38 μg/mL for cyanidin 3-glucoside, cyanidin 3, 5-diglucoside, pelargonidin 3-glucoside, and pelargonidin 3, 5-diglucoside, respectively. These results demonstrate that the method used in this study is suitable for the determination of each anthocyanin compound found in the samples.

3.1.2. Determination of anthocyanin in pomegranate extract [mg/100 g edible portion (e.p.)]

The anthocyanin compositions in pomegranate extract were identified and determined by comparing the peak areas of each anthocyanin compound in the sample with the standards. As shown in the HPLC analysis, the RTs for cyanidin 3-glucoside, cyanidin 3, 5-diglucoside, pelargonidin 3-glucoside, and pelargonidin 3, 5-diglucoside were recorded at 7.738, 7.560, 7.831, and 7.510 min, respectively, based on the reference standards. Based on Table 1, four anthocyanins were identified in the sample which comprised cyanidin 3-glucoside, cyanidin 3, 5-diglucoside, pelargonidin 3-glucoside, and pelargonidin 3, 5-diglucoside, in which the most abundant peak was attributed to cyanidin 3-glucoside with a concentration of 31.60 (12.48) mg/100 g e.p. The total content of anthocyanins quantified in the pomegranate extract sample accounted for approximately 0.15% of the aqueous solution.

3.2. In-vitro studies

3.2.1. Determination of safe dosages for morphine sulfate and pomegranate extract concentrations

In this study, the determination of safe dosages for morphine sulfate and pomegranate extract concentrations in the U-87 cell line were examined using the MTT assay. The assessment of their cytotoxic limits was important to determine the range of safe dosages required for use in the subsequent experiments. As shown in Fig. 1a and b, morphine sulfate and pomegranate extract induced cell death at IC50 concentration values of 1.60 (0.06) μg/mL and 0.44 (0.04) mg/mL, respectively. Hence, further experiments in this study were performed using safe dosage concentrations of 1.25, 0.625, 0.313, and 0.156 μg/mL for morphine sulfate and 0.25, 0.125, 0.0625, 0.0313, and 0.0156 mg/mL for pomegranate extract, respectively.

3.2.2. Regulations of MORs and cAMP in U-87 cell line induced by morphine sulfate and pomegranate extract

The changes in MORs and cAMP concentrations based on the differential effects of morphine and pomegranate extract in the U-87 cell line were examined in this study. Based on Fig. 2 (a), morphine concentrations at 1.25, 0.625, 0.313, and 0.156 μg/mL corresponded to an increasing level of MORs concentrations by 60%, 85%, 83%, and 87%, respectively, at 3 h as compared with the control group. These results were anticipated as morphine was thought to induce regulations of MORs in the U-87 cells, an opioid-expressing cell line, even at low concentrations as compared to the control.

Table 1

| Recovery (%) | Cyanidin 3-glucoside | Cyanidin 3,5-diglucoside | Pelargonidin 3-glucoside | Pelargonidin 3,5-diglucoside |
|--------------|----------------------|--------------------------|-------------------------|-----------------------------|
| Range (μg/mL) | 100.73 (4.77)        | 102.31 (10.84)           | 101.67 (8.58)           | 100.78 (6.83)               |
| Correlation coefficient (r²) | 0.99                 | 0.99                     | 0.99                     | 0.99                        |
| Limits of Detection (μg/mL) | 3.32                 | 11.36                    | 11.32                   | 5.40                        |
| Limits of Quantification (μg/mL) | 10.05               | 34.43                    | 34.29                   | 16.38                       |
| Retention time (minutes) | 7.74 (0.02)          | 7.56 (0.01)              | 7.83 (0.03)             | 7.51 (0.01)                 |
| Concentrations in pomegranate extract (mg/100 g e.p) | 31.60 (12.48)        | 35.31 (14.08)            | 3.60 (6.97)              | 5.30 (8.28)                 |

Data represent mean (SD) of two independent experiments.
According to Eisinger et al. [29], chronic morphine exposure treatment inhibits the internalization and desensitization of the opioid receptor, thereby causing the MORs to be in an active state. This observation was in line with a previous study whereby cells were induced with 1.25 and 0.625 μg/mL of morphine and the MORs concentrations increased significantly \( p < 0.05 \) by 73% and 23%, respectively, from 3 to 48 h, therefore indicating the development from acute to chronic morphine tolerance [20]. Subsequently, the functionality of different concentrations of morphine on MORs concentration levels in U-87 cells was also determined based on the quantification of cAMP. As a GPCR constituent, MORs initiate a signalling event that inhibits adenylase cyclase and subsequently leads to the decrease in intracellular cAMP upon activation [20].

Cells treated with morphine concentrations of 1.25, 0.625, and 0.313 μg/mL correspondingly increased \( p < 0.05 \) the levels of cAMP by 72%, 96%, and 99%, respectively, from 3 to 48 h (Fig. 2 (b)). Additionally, the increasing levels of cAMP from 3 to 48 h indicated the development of acute and chronic opioid tolerance in U-87 cells [20]. Subsequently, concentrations of 1.25, 0.625, and 0.313 μg/mL were regarded as optimum dosages for morphine in cAMP regulations. Besides, the decreasing concentration level of cAMP down to 0.156 μg/mL indicated that the acute morphine state had been gradually wearing off throughout the 48 h-duration and was not able to prolong the chronic morphine state [29].

On the other hand, concentrations of pomegranate extract at 0.25, 0.125, 0.0625, 0.0313, and 0.0156 mg/mL increased the MORs concentration levels by 46%, 65%, 58%, 84%, and 85%, respectively, as compared to the control group at 3 h (Fig. 2 (c)). Interestingly, it was also observed that pomegranate extract could induce the regulation of MORs in the U-87 cell line even at low concentrations as compared to the control, untreated cells. It is important to note that due to their antioxidants and polyphenol content, natural sources have the potency to modulate pain neurotransmitters and addiction pathways [30], thereby acting antagonistically with opioid agonists such as morphine.

Additionally, pomegranate extract concentrations at 0.25, 0.125, and 0.063 mg/mL showed an antagonistic regulation of MORs as compared with morphine treatment (Fig. 2 (c)). These extract concentrations decreased \( p < 0.05 \) MORs concentration levels by 93%, 92%, and 97%, respectively, indicating that these concentrations were able to reduce chronic morphine tolerance from 3 to 48 h. On the other hand, pomegranate extract concentrations of 0.031 and 0.016 mg/mL at 48 h resulted in increasing \( p < 0.05 \) levels of MORs by 43% and 39%, respectively. These results indicate that higher concentrations of pomegranate extract could significantly reduce the MORs concentration levels while lower concentrations of pomegranate extract could lead to the development of tolerance and dependence as shown in Fig. 2 (c). This observation was also noted by Jamil et al. [30], in which the authors showed that the mitragynine compound derived from *Mitragyna speciosa* produced different regulatory effects on MORs at different concentrations using SK-N-SH cells. In contrast, the authors reported that higher concentrations of mitragynine led...
to the development of dependence as opposed to lower concentrations identified in this study.

The functionality of different concentrations of pomegranate extract on the regulation of MORs in U-87 cells was also determined by the quantification of cAMP. It was previously observed that the exposure to chronic morphine led to increasing MORs concentration levels, in which subsequent cAMP levels were detected [30]. Hence, it is thought that pomegranate extract was able to decrease the levels of cAMP to reduce the chronic morphine condition at 48 h. Based on Fig. 2 (d), pomegranate extract concentrations of 0.063 and 0.125 mg/mL (p < 0.05) correspondingly decreased the levels of cAMP by 79% and 82% respectively, at 48 h. Additionally, it was observed that although the MORs concentration level at 0.25 mg/mL could act antagonistically to morphine, the functionality effect due to the increasing level of cAMP suggest that this concentration may act as an agonist similar to morphine (Fig. 2 (d)).

Based on these results, morphine and pomegranate extract concentrations at 0.625 μg/mL and 0.125 mg/mL, respectively, were chosen as optimum dosages as these concentrations acted as agonist and antagonist partners in U-87 cells for the corresponding MORs and cAMP levels. Additionally, these optimum dosages were subsequently used for further experiments in this study.

3.2.3. Effects of co-treatment of morphine sulfate and pomegranate extract on MORs and cAMP regulations

The results depicting the relationship between morphine and pomegranate extract at 48 h for chronic morphine exposure are shown in Fig. 3a and b. The co-treatment of morphine and pomegranate extract was performed to observe the potency of the extract in reducing the levels of MORs and cAMP as compared to morphine alone. As shown in Fig. 3(a), treatment with naloxone resulted in the increase of MORs compared to treatment with methadone and pomegranate extract in the U-87 cell line which showed an increment of up to 11% and 29%, respectively. Meanwhile, co-treatments of morphine with pomegranate extract and morphine with methadone showed a reduction in the concentration levels of MORs by 50% and 29%, respectively, as compared to treatment with morphine alone.

The effect of the interaction between pomegranate extract and morphine on cAMP concentrations at 48 h is shown in Fig. 3(b). The co-treatments of morphine with pomegranate extract and morphine with methadone showed a reduction in cAMP concentration levels by 8% and 5%, respectively. Similarly, treatment with naloxone showed a surge in the level of cAMP as compared to treatment with methadone, pomegranate extract, and morphine in the U-87 cell line which showed increases of up to 26%, 30%, and 22%, respectively. Therefore, pomegranate extract concentration of 0.125 mg/mL had significantly decreased the MORs and cAMP concentration levels as compared to treatment with morphine alone.

Fig. 3. Co-treatment study of morphine and pomegranate extract on (a) MORs and (b) cAMP stimulations at 48 hours incubation. Data represent the mean (SD) of three independent experiments. One-way ANOVA, *p < 0.05 (Bonferroni’s test: compared with control) [Mor: morphine, Mor + Nal: morphine treated with naloxone, Mor + Met: morphine treated with methadone, Mor + PE: morphine treated with pomegranate extract].

3.3. In-vivo studies

3.3.1. Effects of morphine and pomegranate extract on bodyweight changes

The results of the changes in mean bodyweight during pre- and post-treatment of Sprague Dawley male rats are shown in Table 2. Overall, an average decrease in bodyweights by 0.95% and 15.4%, respectively, was observed for the acute and chronic morphine groups investigated in this study. Meanwhile, the bodyweight changes for morphine with pomegranate extract treatment in acute morphine exposure showed an increment by 7.74% as compared to chronic exposure, in which the bodyweight was reduced slightly by 1.48%. In contrast, morphine treatment with methadone showed a reduction in bodyweight by 14.60% (p < 0.05) and 7.80% for both acute and chronic morphine groups, respectively. The oral administration of pomegranate extract (600 mg/kg) to the rats for 7 days indicated that this dosage did not cause any adverse effects toward the bodyweight changes in rats and was presumed to be safe for consumption.

3.3.2. Effects of morphine and pomegranate extract based on the MWM study

Based on the results obtained for the acute and chronic morphine-induced animal experiments performed in this study, the prolonged morphine-induced rats treated with pomegranate extract showed a significant reduction in the total swimming distance (cm) (Fig. 4a). On the other hand, acute and chronic morphine-induced groups showed an increment of 29% and 74%, respectively, for the total swimming distance (cm) taken to find the platform as compared to the group treated with pomegranate extract (Fig. 4(a)). Thus, all morphine-induced groups showed an effect on locomotor activity and spatial memory as evidenced by the MWM experimental results in this study.

In contrast, the escape time latency (s) among the acute and chronic morphine-induced groups showed a significant reduction by 38% for prolonged morphine treatment (Fig. 4b). On the other hand, acute and chronic morphine-induced groups treated with pomegranate extract showed an increment of 16% and 42%, respectively, in the escape time latency (s) taken to find the platform. These results indicate that escape time latency (s) was the shortest for rats treated with pomegranate extract, thereby suggesting the formation of reference memory in these rats as compared to the rats treated with morphine.

Fig. 4. Effects of morphine and pomegranate extract on MWM study. (a) Total swimming distance (cm) and (b) Escape time latency (s) (Fig. 4(a)). Thus, all morphine-induced groups showed an effect on locomotor activity and spatial memory as evidenced by the MWM experimental results in this study.
3.3.3. Effects of morphine and pomegranate extract on CREB and BDNF levels in serum of rats

The CREB and BDNF levels in the serum of rats for acute and chronic morphine-induced groups are shown in Fig. 5(a) and (b). The serum CREB level in the chronic morphine-induced group showed an increment of 68% compared to the acute morphine-induced group. This result was in agreement with a study by Sharma et al. [31], in which the authors revealed that the exposure of chronic morphine eventually increased the expression of CREB in serum. On the other hand, serum CREB levels in rats treated with methadone and pomegranate extract exhibited a reduction of 41% and 37%, respectively, compared to rats treated with morphine only for the chronic morphine-induced treatment. On the other hand, the serum BDNF level in rats treated with methadone and pomegranate extract showed an increment of 11% (p < 0.05) and 26%, respectively, compared to rats injected with morphine in the chronic morphine-induced treatment group. This finding was also consistent with a study by Zhang et al. [32], in which significant increases in serum BDNF levels in heroin-dependent patients were observed approximately one month after heroin cessation.

3.3.4. Correlations between MWM study observations with serum CREB and BDNF levels in rats

In this study, serum levels of CREB and BDNF in the acute and chronic morphine-induced treatment groups showed a positive correlation with the MWM results (data not shown). However, only the serum levels of CREB and BDNF in chronic morphine-induced treatment groups revealed a fair correlation in their total swimming distance (cm) (r = 0.24, p = 0.12) and time latency (s) (r = 0.22, p = 0.15), respectively. No significant difference (p < 0.05) was observed in the relationship between the serum levels of CREB and BDNF in acute and chronic morphine groups with the MWM study results. However, this study revealed that the memory impairment of morphine-induced rats with acute and chronic morphine dosages was improved based on the increased spatial memory and serum levels of CREB and BDNF upon treatment with pomegranate extract.

4. Discussion

The effects of anthocyanin derived from pomegranate extracts on the alterations to the opioid signalling pathway consisting of the MORs-cAMP/CREB-BDNF proteins were successfully examined in this study. The pomegranate fruit contains a rich source of compounds with antioxidant properties, of which a majority is present in the juice as anthocyanins [33]. Although anthocyanin has not been previously studied as an alternative form of non-opioid substitution therapy, previous studies have substantiated the benefits of anthocyanin which include its analgesic [34] and antinociceptive [35,36] effects as well as its use in neurodegenerative diseases [37,38]. Additionally, pomegranate-derived anthocyanin with a concentration of 0.15% in the present study was shown to be

Table 2

| Groups      | Pre-treatment weight of rats (g) | Post-treatment weight of rats (g) | F-statistics (df) | p-value |
|-------------|----------------------------------|-----------------------------------|-------------------|---------|
| **Acute groups** |                                  |                                   |                   |         |
| Control     | 338.25 (48.22)                   | 337.50 (22.52)                    | 0.05 (3)          | 0.97    |
| Mor         | 343.00 (31.08)                   | 339.75 (31.49)                    | 0.12 (3)          | 0.91    |
| Mor + Met   | 349.75 (26.89)                   | 298.75 (25.79)                    | 3.69 (3)          | 0.04*   |
| Mor + PE    | 357.67 (49.69)                   | 387.67 (53.26)                    | –3.23 (3)         | 0.08    |
| **Chronic groups** |                                  |                                   |                   |         |
| Control     | 376.50 (49.14)                   | 379.75 (36.76)                    | –0.32 (3)         | 0.77    |
| Mor         | 374.25 (63.03)                   | 316.75 (35.79)                    | 1.35 (3)          | 0.27    |
| Mor + Met   | 325.25 (15.65)                   | 299.75 (28.98)                    | 1.51 (3)          | 0.23    |
| Mor + PE    | 406.00 (99.68)                   | 400.00 (68.74)                    | 0.27 (3)          | 0.82    |

Data represent the mean (SD) of three independent experiments. Paired T-test, p < 0.05.

[Mor: morphine, Mor + Met: morphine treated with methadone, Mor + PE: morphine treated with pomegranate extract]. Vehicles administered: Mor (solution), Met (solution), Nal (normal saline (solution)) PE (solution).
methadone, within and between groups [Mor: morphine, Mor + PE: morphine treated with pomegranate extract].

In this study, the safe dosage range for morphine sulfate at 0.625 µg/mL determined was shown to cause several alterations to the MORs and cAMP protein levels. This was supported by Eisinger et al. [29], which further observed a significant increase in the levels of MORs at 0.625 µg/mL for the treatment of chronic morphine, in which this concentration was thought to inhibit internalization and desensitization of the receptor and cause MORs to be in an active state. Interestingly, pomegranate extract was shown to reduce the MORs protein level (p < 0.05) at the higher concentrations while lower concentrations could lead to the development of tolerance and dependence. Therefore, it is evident that natural sources have the potency to modulate pain neurotransmitters and addiction pathways due to the presence of antioxidants and polyphenol content [29].

Several studies by Ibrahim et al. [11], Adnan et al. [12], and Jamil et al. [28] have highlighted the potential of *P. dactylifera*, thymoquinone from *N. sativa*, and mitragynine from *M. speciosa* as non-opioid treatments in chronic opioid dependence. Interestingly, *P. dactylifera* extract was described as a calcium channel antagonist [11], while thymoquinone was identified as an opioid receptor-stimulating compound with a 45% ligand displacement of MORs [42]. Additionally, mitragynine was recognized as a substance with opioid-like effects in *in-vivo* studies [43]. In contrast, the anthocyanin compound in pomegranate extract has never been previously investigated regarding their interaction with receptor ligands of MORs. Nevertheless, the C ring structure of anthocyanin carries a positive charge with an electron deficiency, thereby conferring antioxidant properties when the structure donates a hydrogen atom and an electron to deactivate the free radicals in the body [44]. On the other hand, the chemical structure of morphine acts as an agonist or becomes chemically active at its receptor due to its phenolic and secondary alcoholic functional groups at C3 and C6, respectively [46].

Theoretically, the chemical structures of anthocyanin and morphine have a carbon ring that can be activated to produce health benefits. For instance, it is evident that pomegranate-derived anthocyanin may have potency due to its affinity to MORs, thereby regulating the opioid receptors based on the adaptive changes in MORs and cAMP levels. On the other hand, the potency of pomegranate-derived anthocyanin *in-vivo* has also been investigated, in which anthocyanin was shown to be involved in the activation of the ERK-CREB-BDNF protein pathway in neurodegenerative studies [37,38]. In opioid addiction and dependence, the drugs are able to take over the normal learning process and memory systems through direct pharmacological effects on multiple neurotransmitter systems, thereby profoundly influencing the learning process and memory circuits [7].

The MWM study is a measure of hippocampally-dependent spatial navigation and a validity method for reference memory using animal models [45]. This method has been utilized to identify the long-term potentiation (LTP) and the function of opioid agonist receptors in opioid studies [46]. Based on the MWM experimental results, rats treated with pomegranate extract showed a decrease in the total swimming distance (cm) and total time latency (s) by 60% and 42%, respectively, compared to the morphine treatment group, in the chronic groups. These results suggest that the treatment with pomegranate extract could enhance learning and behavioral memory indicated that the rats exhibited the formation of reference memory as compared to the rats treated with morphine alone.

In the learning and memory processes, LTP is one of the main cellular mechanisms in the brain that facilitate information storage [47]. There are various signaling pathways associated with the synthesis of new proteins in the LTP phase which include cAMP-dependent PKA, protein kinase B, PKC, and ERK [48,49]. Previously, Socci et al. [50] and Angeloni et al. [51] discovered that flavonoid could induce the activation of CREB and subsequent development of BDNF in brain neurons. Eventually, these cellular events led to the increased translation of specific effectors and synaptic strength which enabled the induction of neuronal dendrite outgrowth through the ERK and BDNF signalling pathway [52]. This finding was also supported by Maher et al. [53], in which the authors showed that flavonoids could improve object recognition and LTP through the activation of the ERK-CREB-BDNF pathway.
The results in this study were also consistent with a previous study by Ilyas et al. [37], in which the authors demonstrated that anthocyanin or flavonoid content improved memory function in rats. The capability of anthocyanin to reverse memory declines also depends on its potential interaction within the cellular and molecular regions of the brain that is responsible for memory [54]. Generally, the storage of short-term memory requires functional alterations in the strength of pre-synaptic connections [55], while the storage of long-term implicit and explicit memory involves the synthesis of new proteins and growth of new synapse connections [56]. The positive effects of pomegranate extract based on the MWM results obtained in this study indicate its capability to synthesize new proteins that increase memory processing [54], thereby reversing the decline in memory.

Apart from improving cognitive impairment, the most significant health benefit of anthocyanin is for the protection of the brain, in which it is able to traverse the blood–brain barrier (BBB). Anthocyanin is a highly water-soluble molecule and is primarily absorbed in the form of intact glycosidic molecules that can reach the circulatory system within 6–20 min and is subsequently excreted into the urine [57]. A majority of the anthocyanins do not undergo a detailed metabolic process as this compound has been detected in the form of intact glycosides in both urine and plasma samples [58]. Additionally, Vanzo et al. [58] observed that rats fed with an anthocyanin-based diet for 15 days were shown to have anthocyanin in their stomach tissue, jejunum, liver, kidney, and brain within 10 min after digested into the stomach. Besides, Kalt et al. [59] detected anthocyanin in several regions of the brain consisting of the cortex, cerebellum, and hippocampus after the consumption of an anthocyanin-based diet for 28 days.

Based on the previous studies on anthocyanin administration and its effects on the body, it is evident that pomegranate-derived anthocyanin extracts used in this study improved the performance of memory tasks in the brain in-vivo within 7 days. Specifically, the use of pomegranate-derived anthocyanin extract was shown to improve memory impairment based on the MWM experimental results and increase the serum levels of CREB and BDNF in rats exposed to acute and chronic morphine-induced treatments performed in this study. These observations have not been previously reported in any study and therefore, substantiate the view that pomegranate-derived anthocyanins may have a central neuroprotective role for learning and memory disorders caused by opioid addiction and dependence.

5. Conclusion

In conclusion, the effects of pomegranate extract on the proteins involved in the opioid signalling pathway which include MORs, cAMP/CREB, and BDNF have been successfully investigated using in-vitro and in-vivo experiments established in this study. To the best our knowledge, this is the first study performed to investigate the potency of pomegranate-derived anthocyanin extract as an alternative non-opioid substitution therapy. The findings of this study could potentially serve as preliminary data for future studies on drug development from natural resources to identify other key proteins and thus, develop better intervention strategies to combat opioid dependence in individuals.

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Conflict of interest

None.

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