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Original article

Secondary metabolites isolated from *Pinus roxburghii* and interpretation of their cannabinoid and opioid binding properties by virtual screening and *in vitro* studies

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ABSTRACT

Pinus roxburghii is highly popular as a potent analgesic and anti-inflammatory agent; however its exact mechanism of action was not fully elucidated. We aimed to interpret the analgesic and anti-inflammatory activity of the total ethanol extract of *Pinus roxburghii* bark (PRE) and its isolated compounds by both *in silico* molecular modelling and *in-vitro* cannabinoid and opioid binding activities evaluation for the first time. Comprehensive phytochemical investigation of PRE resulted in the isolation of sixteen compounds that were fully elucidated using ^{1}H NMR and ^{13}C NMR. Four of which namely 1,3,7-trihydroxyxanthone (**1**), 2,4,7-trihydroxyxanthone (**2**), isopimaric acid (**9**) and 3-methoxy-14-serraten-21-one (**10**) were first to be isolated from PRE. *In silico* molecular modelling was done using Accelry's discovery studio 2.5 on the cannabinoid receptor (CB1) and the different opioid receptors (*mu, kappa and delta).* Results showed that the different isolated constituents exhibited variable degrees of binding with the different examined receptors that undoubtedly explained the observed analgesic and anti-inflammatory activity of PRE. Thus *in vitro* evaluation of cannabinoid (CB1, CB2) and opioid (μ, κ, δ) binding activities for the isolated compounds was done. PRE and ursolic acid (**11**) showed a good CB1 receptor binding activity with 66.8 and 48.1% binding, respectively. Isopimaric acid (**9**) showed good CB2 and *mu* receptors binding activity estimated by 58.1 and 29.1% binding, respectively. Meanwhile, querectin-3-*O*-rhamnoside (**7**) exhibited a moderate κ-opioid receptor activity showing 56.0% binding. Thus, PRE could offer a natural analgesic and anti-inflammatory candidate through the synergistic action of all its components.

1. Introduction

Pain and inflammation are considered as two severe discriminate conditions that are closely associated. Pain can be defined as an annoying sensation that is always accompanied by real or probable tissue destruction (Eisenberger and Lieberman, 2004). Meanwhile inflammation is the natural tissue defence mechanism to any exterenous matter as well as to injury resulting in the migration of the leucocytes and antibodies to the affected parts with concomitant appearance of swelling and oedema (Stankov, 2012). Synthetic analgesics and anti-inflammatory agents can reduce symptoms but unfortunately they sparked a lot of undesirable side effects owing to their nonselective attitude (Tapiero et al., 2002). Thus the need for naturally occurring relatively safer candidates for the alleviation of pain and inflammation is felt mandatory worldwide.

Genus *Pinus,* which comprises of nearly about 120 species, spreads along the temperate regions of the Northern Hemisphere. It is known as Chir Pine and characterized by being a tall tree. It is used as a folklore medicine in the alleviation of bronchial disorders, asthma, dermal diseases as well as convulsion, hepatic diseases and spine, piles, toothache, earache, scabies, gonorrhea and ulcers (Shuaib et al., 2013; Kaushik et al., 2014). Moreover, different parts of the plant *viz.* resin, oil, needles, bark, wood and even

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seeds, had been used in traditional medicine to treat different ailments. However, local application of the resin is beneficial to treat boils meanwhile its oral administration could effectively relief gastric trouble (Rajbhandari, 2001; Narayan and Manandhar, 2002). Owing to its popular anti-inflammatory properties, it is widely employed by Native Americans to alleviate rheumatism. The wood oil extracted from *P. roxburghii* is used as diuretic, haemostatic and neural tonic (Puri et al., 2011). In a previous publication, the essential oil of *P. roxburghii* bark showed powerful anti-inflammatory activity (Labib et al., 2017).

However, the resin ointment from the bark showed high efficacy in curing dermal burns and cracks and other skin diseases (Kaushik et al., 2013) in addition to its usage in Himalayan region as emollient, stimulant, antiseptic, anthelmintic, liver, tonic, diaphoretic and diuretic (Rashid et al., 2015). Previous reports had showed alcoholic bark extract to possess analgesic, anti-inflammatory, anti-convalescent and anti-diabetic activities (Kaushik et al., 2012, 2015). Different classes of secondary metabolites have been isolated from the bark including polyphenolics such as flavonoids, xanthones, tannins in addition to sugars (Shuaib et al., 2013). This huge variety of phytoconstituents and multiple ethnopharmacological uses had attracted our attention to carry out a comprehensive study regarding the chemistry and pharmacology of *Pinus roxburghii* cultivated in Egypt.

Herein, we reported the isolation and structural elucidation of (**1**–**16**) compounds from the bark. Besides, molecular modelling studies of the isolated compounds in the active sites of opioid and cannabinoid receptors were done in an effort to explore the exact mechanism of action beyond the ethnopharmacological popularity of the bark as an analgesic and anti-inflammatory. Furthermore, *in vitro* studies were done for the first time to ascertain their cannabinoid and opioid binding properties.

2. Materials and methods

2.1. General experimental procedures

Bruker model AMX 400 NMR spectrometer operating on a standard pulse system used for measuring ^{1}H and ^{13}C NMR spectra. The instrument ran at 400 MHz in ^{1}H and 100 MHz in ^{13}C. $CDCl_3$ and CD_3OD were used as solvents whereas TMS was used as an internal standard. HRMS were obtained on a Micromas Q-T of Micro mass spectrometer. Column chromatographic separation was done on silica gel (60–120 mesh, Merck) and Sephadex LH 20. Thin layer chromatography precoated aluminum sheets [silica 60 F254, 0.25 mm (Merck, Darmstadt, Germany)] were used to monitor fractions eluted from column chromatography. Visualization of TLC plates was achieved using UV lamp and vanillin sulphuric acid spray reagent. All used solvents were of analytical grade.

2.2. Plant material

Pinus roxburghii Sarg. (*syn. Pinus longifolia*) bark, Family Pinaceae was collected from El- Orman Botanical Garden on April 2014 and authenticated by Mrs. Terease Labib, Consultant of Plant Taxonomy at Ministry of Agriculture and El-Orman Botanical Garden and National Gene Bank, Giza, Egypt. A voucher specimen was kept in the Herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt (P-PR-7).

2.3. Extraction and isolation

The powdered air dried *P. roxburghii* bark (2 kg) was extracted with aqueous ethanol (4 × 2 L) till exhaustion to afford 110 g dried extract (PRE). It was then fractionated using silica gel vacuum liquid chromatography (VLC) starting with *n*-hexane and increase the polarity using EtOAc followed by MeOH to give 12 subfractions which were monitored using TLC and pooled together to give 4 main fractions: A (eluted with *n*-hexane 100%; 14.38 g), B (eluted with Hex.: EtOAc; 1:9 & EtOAc 100%; 13.75 g), C (eluted with EtOAc: MeOH, 2:98 &10:90; 21.2 g) and D (eluted with EtOAc: MeOH, 50:50 & MeOH 100%; 14.4 g). Fraction C was applied on top of silica gel column and eluted with EtOAc: MeOH to afford 41 fractions. Fractions 22, 23, 24; eluted with 100% EtOAC were pooled together and were applied on sepahdex column and eluted with MeOH to afford compounds **3** (9 mg) and **15** (6 mg). Fractions 26, 27 & 28; eluted with 20% EtOAC: MeOH were fractionated over Sephadex LH 20 and eluted with MeOH to afford compounds **5** (6 mg), **9** (5 mg) and **14** (10 mg). Fraction 29; eluted with 40% EtOAC: MeOH were fractionated over Sephadex LH 20 and eluted with MeOH to afford compounds **4** (5 mg). Fraction 30; eluted with 40% EtOAC: MeOH were fractionated over Sephadex LH 20 and eluted with MeOH to afford compounds **1** (8 mg), **2** (5 mg) and **13** (8 mg). Fractions 31, 32 & 33; eluted with 60% EtOAC: MeOH were fractionated over Sephadex LH 20 and eluted with MeOH to afford compounds **7** (4 mg), **11** (10 mg) and **12** (3 mg). Fractions 34, 35 & 36; eluted with 80% EtOAC: MeOH were fractionated over Sephadex LH 20 and eluted with MeOH to afford compounds **10** (8 mg). Fractions 37–43; eluted with 100% MeOH were fractionated over Sephadex LH 20 and eluted with MeOH to afford compounds **6** (10 mg), **8** (6 mg) and **16** (5 mg).

2.4. Molecular modelling studies

The X-ray crystal structure of cannabinoid receptor CB1 (PDB ID5U09; 2.6 A°) and opioid receptors *mu* (PDB ID 5C1M; 2.1 A°), δ (PDB ID 4EJ4; 3.4 A°), κ (PDB ID 4DJH; 2.9 A°) co-crystallized with their ligands were downloaded from protein data bank (www.pdb.org). Molecular modelling studies were done using Accelry's discovery studio 2.5 (Accelrys®, Inc., San Diego) in accordance to what previously reported (Youssef et al., 2017) and the binding free energies were calculated applying the following equation:

$$\Delta G_{binding} = E_{complex} - (E_{Protein} + E_{ligand}) \quad (1)$$

where;

$\Delta G_{binding}$: The ligand–enzyme interaction binding energy,
$E_{complex}$: The potential energy for the complex of protein bound with the ligand,
$E_{protein:}$ The potential energy of the protein alone and,
E_{ligand}: The potential energy for the ligand alone

2.5. Cannabinoid and opioid receptor assays

The affinity of extract and isolated compounds towards cannabinoid and opioid receptors was carried out according to the published method (Tarawneh et al., 2015).

2.5.1. Cell culture

Human embryonic kidney-293 cells [HEK-293] (ATCC, Manassas, VA) were stably transfected *via* electroporation with full-length human recombinant cDNA (OriGene, Rockville, MD) for cannabinoid receptor subtypes 1 and 2 and human opioid receptors subtypes *(Mu-, Delta-, Kappa-)*. These cells were maintained at 37 °C and 5% CO_2 in a Dulbecco's Modified Eagles' Medium (DMEM) and F-12 HAM nutrient mixture (50/50), supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 1% penicillin-streptomycin, and G418 antibiotic solutions. Membranes for the radioligand binding assays were prepared by scraping the cells in a 50 mM Tris-HCl buffer, followed by homogenization, sonication, and centrifugation for 40 min at 13,650 rpm at 4 °C. These were kept at −80 °C until used for bioassays. Protein concentration

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