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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

was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). The total alcoholic extract PRE and the isolated compounds were run in competition binding assays against both cannabinoid receptor subtypes and all three opioid receptor subtypes (Tarawneh et al., 2015).

2.5.2. Cannabinoid receptors binding assay

Cannabinoid binding took place under the following conditions: 10 μM of each compound was incubated with 0.6 nM [^{3}H] CP 55.940 and 10 μg of CB1 or CB2 membranes for 90 min in a silanized 96-well plate. The reaction was terminated *via* rapid vacuum filtration through GF/B filters presoaked with 0.3% bovine serum albumin (BSA) using a Perkin-Elmer 96-well Unifilter followed by 10 washes with 50 mM Tris-HCl. Plates were read using a Perkin-Elmer Topcount.

2.5.3. Opioid receptors binding assay

Opioid binding assays were performed under the following conditions: 10 μM of each compound was incubated with [3H]-DAMGO (μ), [3H]-U-69,593 (κ), or DPDPE (δ) for 60 min in a 96-well plate. Percent binding was calculated as the average of the triplicate tested at 10 μM. Each sample concentration point of the compounds tested in dose response was in triplicate, and each compound showing activity was tested at least three times. The reaction was terminated *via* rapid vacuum filtration through GF/B filters presoaked with 0.3% bovine serum albumin (BSA) using a Perkin-Elmer 96-well Unifilter followed by 10 washes with 50 mM Tris-HCl. Plates were read using a Perkin- Elmer Topcount. Total binding was defined as binding in the presence of 1.0% DMSO. Nonspecific binding was the binding observed in the presence of 10 μM of the radioactive ligands.

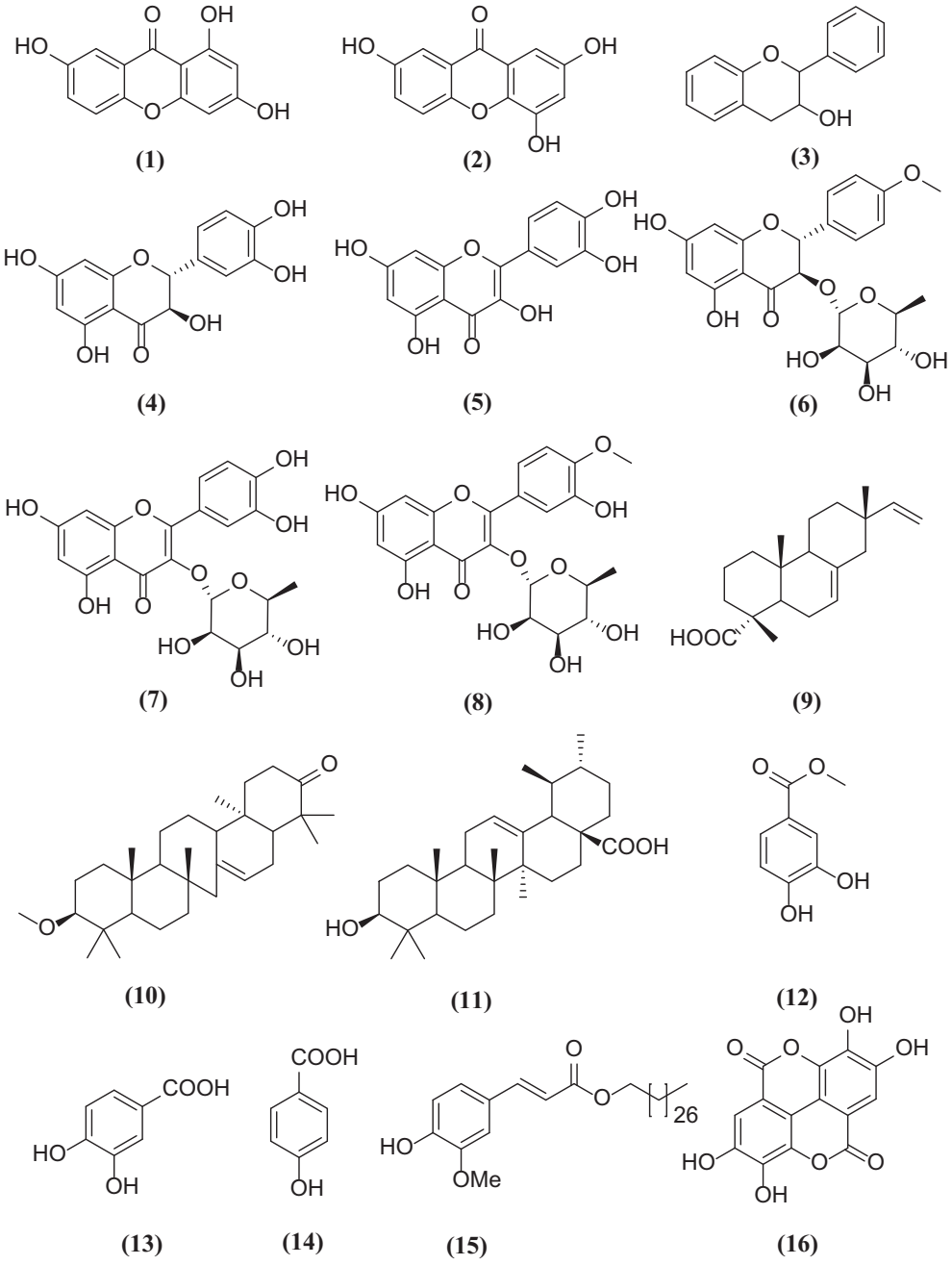

Fig. 1. Isolated phytoconstituents from PRE.

2.5.4. Data analysis

The analysis of data was carried out by using a non-linear curve fit model applying GraphPad Prism 5.04 software (GraphPad, La Jolla, CA) and the Kd value was calculated. General screening was performed using the optimal concentration of membrane with a radioligand concentration ≤to the Kd. Each compound was test at 10 μM in triplicates. The assays were performed as stated above. Specific binding was calculated *via* the subtraction of non-specific binding from total binding. Percent binding was determined using the following equation:

$$\%\text{binding} = 100 - [\text{Compound CPM} - \text{nonspecific CPM}/\text{specific CPM}] * 100 \quad (2)$$

3. Results and discussion

3.1. Secondary metabolites isolated from PRE

Comprehensive phytochemical investigation of PRE resulted in the isolation and structural elucidation of sixteen compounds as illustrated in Fig. 1. These compounds were identified as, two xanthones namely 1,3,7-trihydroxyxanthone (**1**) (Jantan and Saputri, 2012) and 2,4,7-trihydroxyxanthone (**2**) (Jantan and Saputri, 2012); in addition to six flavonoids which are flavan-3-ol (**3**) (Krohn et al., 2009), taxifolin (**4**) (Kim et al., 2012), quercetin (**5**) (Aderogba et al., 2013), 5,7-dihydroxy-4′-methoxy dihydroflavanol-3-*O*-rhamnoside (**6**) (Bilia et al., 1993), querectin-3-*O*-rhamnoside (**7**) (Aderogba et al., 2013) and isorhamnetin-3-*O*-rhamnoside (**8**) (Zhang et al., 2014); one diterpene, isopimaric acid, (**9**) (Piovano et al., 1988); two triterpenes, 3-methoxy-14-serraten-21-one (**10**) (Kutney et al., 1969) and ursolic acid (**11**) (Babalola and Shode, 2013); besides five known phenolic compounds: methylprotocatechuate (**12**) (Dal Picolo et al., 2014), 3,4-dihydroxybenzoic acid (**13**) (Syafni et al., 2012), *p*-hydroxybenzoic acid (**14**) (Chen et al., 2008), octacosyl ferulate (**15**) (Ruan et al., 2007), and ellagic acid (**16**) (Li et al., 1999). The purity of the isolated compounds are indicated from the NMR charts present in the Supplementary data. Noteworthy to mention that compounds 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.

Table 1
Free binding energies (kcal/mol) of the isolated compounds from PRE in the active sites of the cannabinoid and opioid receptors using molecular modelling experiments.

Compound	CB1	Delta [δ]	Mu [μ]	Kappa [κ]
1,3,7-trihydroxyxanthone **(1)**	−31.00	−35.35	−29.13	−29.80
2,4,7-Trihydroxyxanthone **(2)**	−31.62	−35.46	−32.86	−30.08
Flavan-3-ol **(3)**	−34.01	−30.26	−26.73	−28.11
Taxifolin **(4)**	−44.36	−45.41	−38.53	−38.19
Quercetin **(5)**	−41.90	−43.64	−35.54	−36.38
5,7-Dihydroxy-4′-methoxy dihydroflavanol-3-*O*-rhamnoside **(6)**	−60.83	−55.33	−55.46	−56.06
Querectin-3-*O*-rhamnoside **(7)**	−60.44	−54.76	−53.76	−56.25
Isorhamnetin-3-*O*-rhamnoside **(8)**	−64.58	−57.35	−57.43	−54.74
Isopimaric acid **(9)**	−31.60	−29.16	−36.51	−31.67
3-methoxy-14-serraten-21-one **(10)**	−6.96	−42.13	−46.96	−42.81
Ursolic acid **(11)**	−9.21	−39.12	−38.28	−42.52
Methylprotocatechuate **(12)**	−23.64	−25.59	−23.48	−24.71
3,4-dihydroxybenzoic acid **(13)**	−22.18	−28.16	−21.04	−22.43
p-Hydroxybenzoic acid **(14)**	−21.67	−23.04	-18.66	−20.14
Octacosyl ferulate **(15)**	−36.02	−31.97	−34.03	−31.12
Ellagic acid **(16)**	−33.53	−37.65	-34.09	−30.87
[3H] CP 55.940	−55.50	ND	ND	ND
DPDPE	ND	−67.10	ND	ND
[3H]-DAMGO	ND	ND	−61.23	ND
[3H]-U-69,593	ND	ND	ND	−44.41

ND: Not done.

Table 2
Cannabinoid and opioid receptors binding percentages of the phytoconstituents isolated from PRE at 10 μg/mL.

Extract/Compound	CB1	CB2	Delta [δ]	Mu [μ]	Kappa [κ]
PRE	**66.8**	13.5	–	15.2	11.8
1,3,7-trihydroxyxanthone **(1)**	–	6.1	33.8	2.6	–
2,4,7-Trihydroxyxanthone **(2)**	–	–	33.8	2.6	–
Flavan-3-ol **(3)**	–	–	–	–	–
Taxifolin **(4)**	–	2.5	**40.2**	–	8.4
Quercetin **(5)**	–	6.5	14.4	–	27.8
5,7-Dihydroxy-4′-methoxy dihydroflavanol-3-*O*-rhamnoside **(6)**	–	–	–	–	–
Querectin-3-*O*-rhamnoside **(7)**	35.6	–	8.9	3.8	**56.0**
Isorhamnetin-3-*O*-rhamnoside **(8)**	–	6.5	29.5	–	22.0
Isopimaric acid **(9)**	17.3	**58.1**	8.0	**29.1**	25.6
3-methoxy-14-serraten-21-one **(10)**	–	12.2	0.9	–	11.1
Ursolic acid **(11)**	**48.1**	–	18.8	17.7	39.4
Methylprotocatechuate **(12)**	–	–	–	–	–
3,4-dihydroxybenzoic acid **(13)**	–	–	1.4	0.7	17.9
p-Hydroxybenzoic acid **(14)**	11.9	17.3	18.8	6.9	49.4
Octacosyl ferulate **(15)**	–	–	–	–	–
Ellagic acid **(16)**	–	–	22.7	–	20.1

–: Not active.

3.2. In vitro evaluation of the cannabinoid (CB1, CB2) and opioid (μ, κ, δ) receptor binding activity of the alcoholic extract

Management of either pain or inflammation could be better achieved *via* the use of a combined therapy comprises of both anti-inflammatory and analgesic agents (Anilkumar, 2010). There are many targets for both the anti-inflammatory agents as well as the analgesics that mainly include prohibition of prostaglandins synthesis in its two major pathways of COX and LOX. Additionally, stimulation of endogenous opioids production, inhibition of G-protein-mediated signal transduction and interfering with 5-hydroxytryptamine production are among the popular modes of action of potent analgesics (Cashman, 1996; Nalini Sehgal et al., 2011). The opioid and cannabinoid receptors are G-protein coupled receptors and are located mainly within the central nervous system (CNS). Various subtypes of opioid and cannabinoid G protein receptor systems have been recognized; the opioid receptor system involved mainly μ, κ (kappa), and δ (delta) receptors, while the cannabinoid receptor system includes CB1 and CB2 receptors. Agonists of opioid and cannabinoid receptors are known to produce powerful analgesia and have been explored pharmacologically for the treatment of various neuropathic pains (Manzanares et al., 2006). Thus, in this context we examined the analgesic activity *via* acting on both the cannabinoid and opioid receptors. The alcoholic extract was evaluated for the cannabinoid (CB1, CB2) and opioid (μ, κ, δ) receptor assays and it showed a promising CB1 activity with 66.8% percent binding at 10 μg/mL in addition to a mild μ, κ activity manifested by 15.2 and 11.8% binding, respectively.

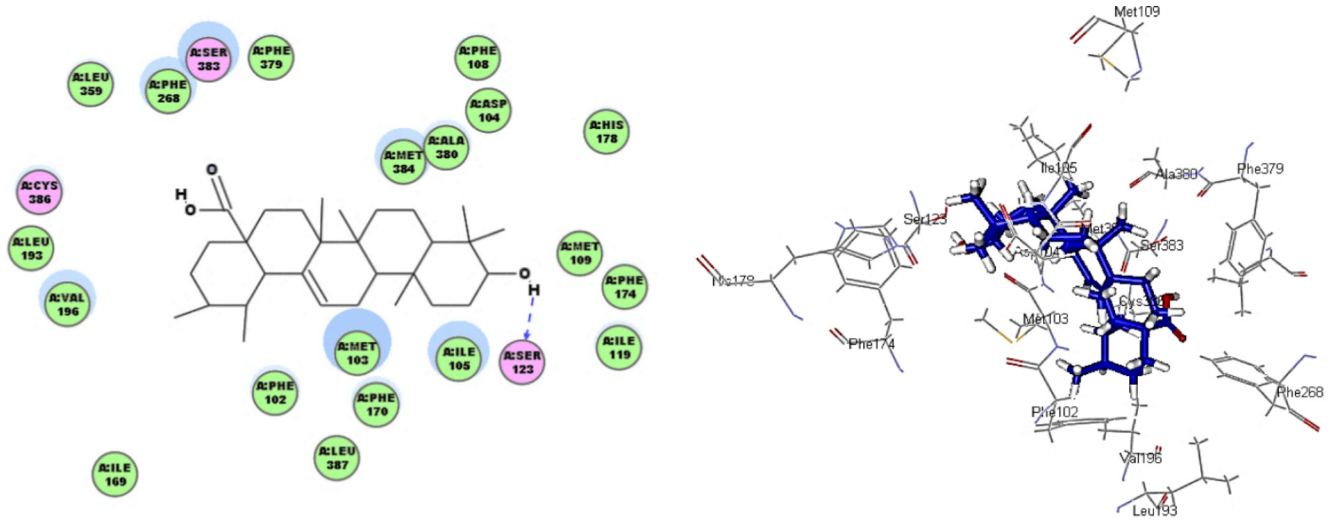

Fig. 2. 2D and 3D binding of ursolic in the active site of CB1 receptor.

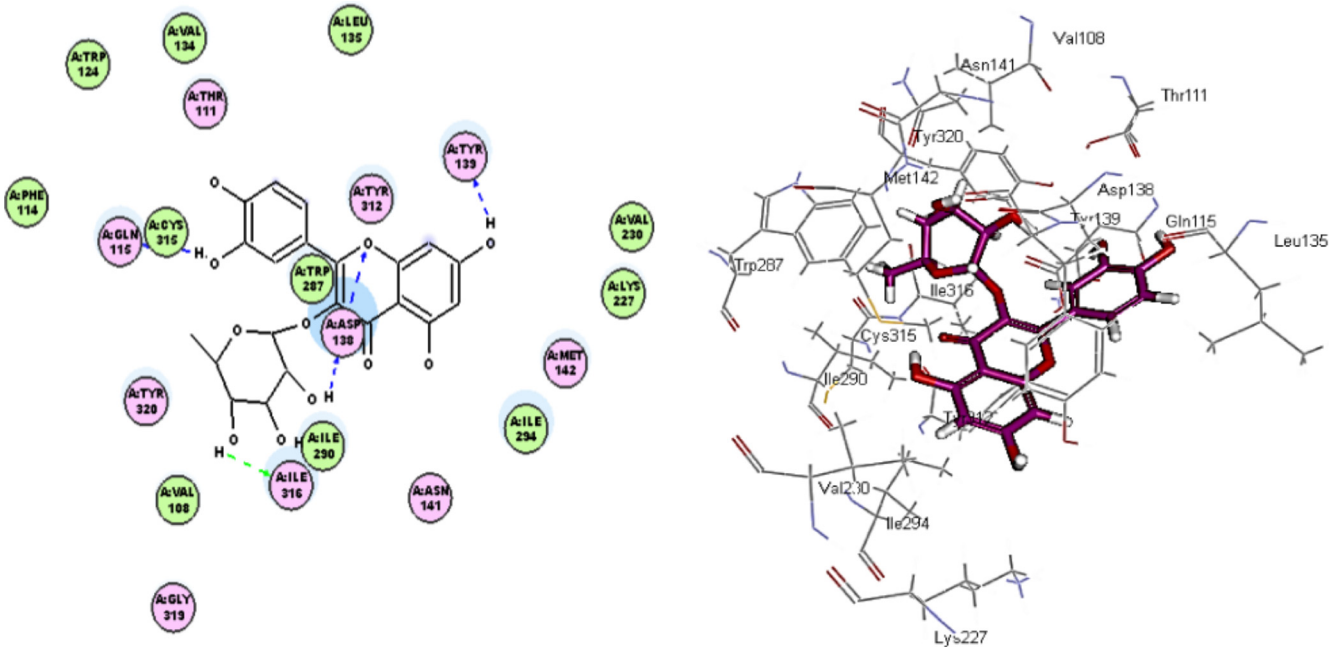

Fig. 3. 2D and 3D binding of quercetin-3-*O*-rhamnoside in the active site of *kappa* receptor.

3.3. Molecular modelling studies of all of the isolated compounds within the active sites of the cannabinoid (CB1) and opioid (μ, κ, δ) receptors

Moreover molecular modelling studies of all of the isolated compounds within the active sites of the cannabinoid (CB1) and opioid (μ, κ, δ) receptors was done in an attempt to explore their exact target receptor. Molecular docking was not done on CB2 as it is still unavailable on the Protein data bank (PDB) in its crystallized form. Results displayed in Table 1 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. This encourages us to further proceed in the evaluation of cannabinoid (CB1, CB2) and opioid (μ, κ, δ) binding activities for the isolated compounds using *in vitro* studies.

3.4. In vitro evaluation of the cannabinoid (CB1, CB2) and opioid (μ, κ, δ) receptor binding activity of the isolated compounds

Bioassay was performed as reported in literature and active compound results were recorded in Table 2. Unexpectedly, ursolic acid (**11**) showed a moderate CB1 activity estimated by 48.1% binding although it exhibited weak binding in the molecular modelling studies with free binding energy equals to −9.21 kcal/mol. However by examining its 2D binding mode a lot of hydrophobic interactions were observed with the existing amino acid residues at the active site in addition to one hydrogen bond with Ser123. The difference between the computed values in docking studies and the evaluated binding percentages *in vitro* could greatly relied upon the major differences between the biological medium and the *in silico* experiments and reflects that effect of the biological system to change the binding mode (Fig. 2).

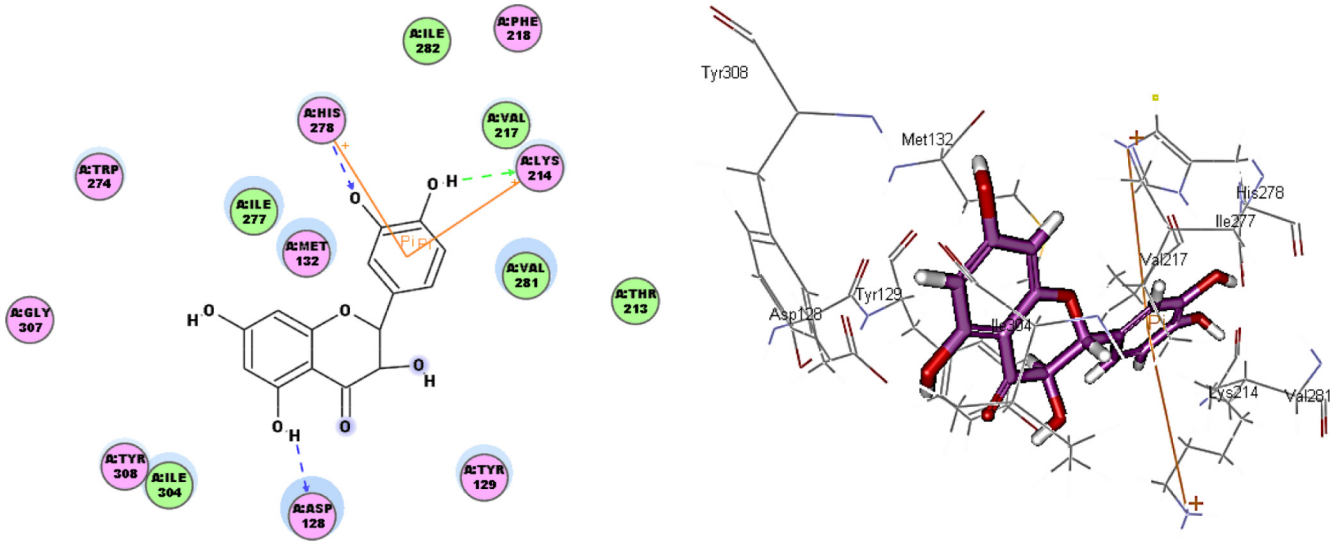

Fig. 4. 2D and 3D binding of taxifolin in the active site of *delta* receptor.

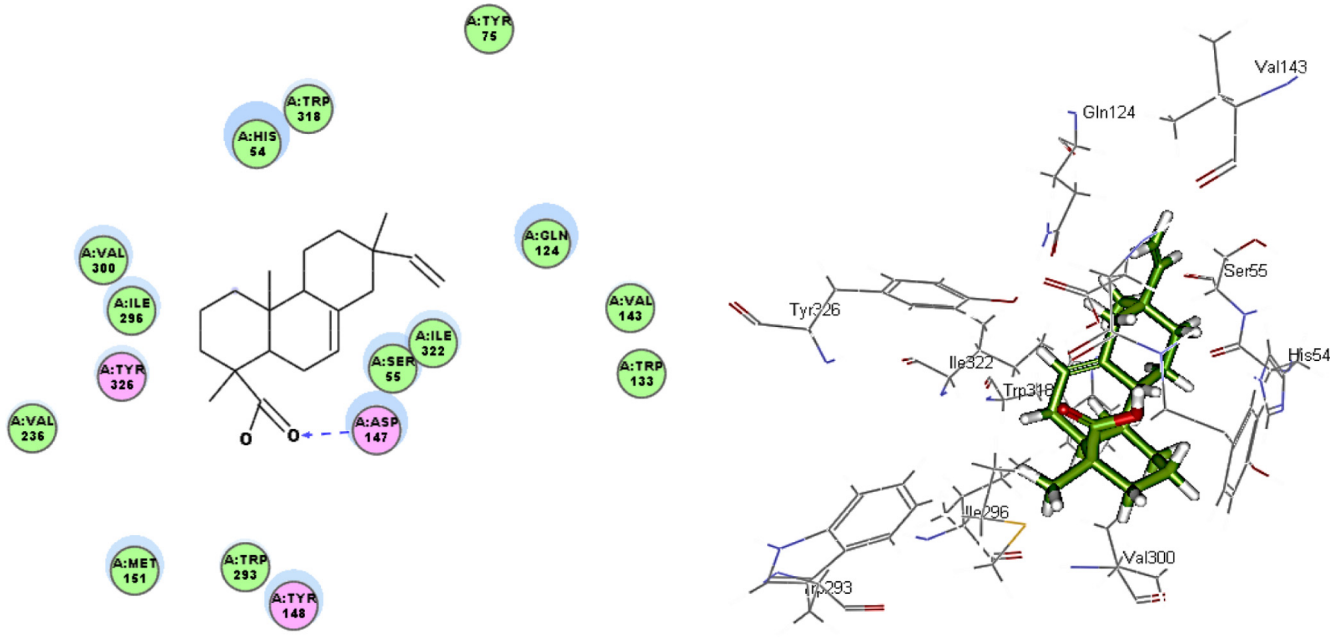

Fig. 5. 2D and 3D binding isopimiric acid in the active site of *mu* receptor.

Meanwhile quercetin-3-*O*-rhamnoside (**7**) showed moderate κ-opioid activity with 56% binding that could be explained in terms of molecular modelling where it displayed the highest binding as evidenced from its free binding energy that was −56.25 kcal/mol. Querectin-3-*O*-rhamnoside (7) exerted four hydrogen bonds at the active site of *kappa* receptor two of which were formed with Asp 138, one with Ile 316 and the last formed with Tyr 139 as showed from its 2D and 3D binding modes displayed in Fig. 3.

However, taxifolin (**4**) showed a good binding with the *delta* receptor as evidenced from the *in silico* study showing a binding free energy of −45.41 kcal/mol that was supported by the *in vitro* study where the compound showed 40.20% binding percentage. Taxifolin **(4)** forms two hydrogen bonds and two π- bonds with His 278 and Lys 214 amino acid residues in addition to a third hydrogen bond with Asp 128 as illustrated in Fig. 4. Moreover, isopimaric acid (**9**) showed good CB2 and *mu* receptors binding activity estimated by 58.1 and 29.1% binding, respectively. The binding of the compound on the latter receptor is attributed to the formation of one hydrogen bond between the carbonyl carbon and Asp 147 amino acid in addition to the hydrophobic interaction with the residues present at the active site as revealed in Fig. 5.

Thus both the alcoholic extract and ursolic acid can be utilized as analgesic and anti-inflammatory drugs. While isopimaric acid (**9**) can be used to increase the appetite as it had showed good CB2 activity. Nevertheless, quercetin-3-*O*-rhamnoside (**7**) and *p*-hdroxybenzoic acid (**14**) can be used as peripheral analgesic due to their moderate κ-opioid activity.

4. Conclusion

Comprehensive phytochemical investigation of PRE resulted in the isolation and structural elucidation of sixteen compounds that were classified as: two xanthones, six flavonoids, one diterpene, and two triterpene besides five phenolic compounds. Four compounds namely:1,3,7-trihydroxyxanthone (**1**); 2,4,7-trihydroxyxanthone (**2**); isopimaric acid (**9**) and 3-methoxy-14-serraten-21-one (**10**) were isolated for the first time from the reported plant. 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. However *in vitro* studies manifested that that isopimaric acid (**9**) showed good CB2 activity. Ursolic acid showed moderate CB1 activity Quercetin-3-*O*-rhamnoside (**7**) and *p*-hydroxybenzoic acid (**14**) showed moderate κ-opioid activity. Thus, PRE could offer a relatively safe, natural analgesic and anti-inflammatory candidate through the synergistic action of all its components.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jsps.2017.12.017.

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