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Xanthine oxidase inhibitory activity of natural and hemisynthetic flavonoids from *Gardenia oudiepe* (Rubiaceae) *in vitro* and molecular docking studies

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ABSTRACT

Xanthine oxidase (XO), an enzyme widely distributed among mammalian tissues, is associated with the oxidation of xanthine and hypoxanthine to form uric acid. Reactive oxygen species are also released during this process, leading to oxidative damages and to the pathology called gout. Available treatments mainly based on allopurinol cause serious side effects. Natural products such as flavonoids may represent an alternative. Thus, a series of polymethoxyflavones isolated and hemisynthesized from the bud exudates of *Gardenia oudiepe* has been evaluated for in vitro XO inhibitory activity. Compounds **1**, **2** and **3** were more active than the reference inhibitor, Allopurinol (IC_{50}= 0.25±0.004 µM) with IC_{50} values of (0.004±0.001) µM, (0.05±0.01) µM and (0.09±0.003 µM), respectively. Structure-activity relationships were established. Additionally, a molecular docking study using MOE^{TM} tool was carried out to establish the binding mode of the most active flavones with the enzyme, showing important interactions with its catalytic residues.

These promising results, suggest the use of these compounds as potential leads for the design and development of novel XO inhibitors.

Keywords: *Gardenia oudiepe*, flavonoid, xanthine oxidase inhibition, molecular docking.

1. Introduction

Xanthine oxidase (XO) has been reported as a key enzyme associated with the oxidation of purines hypoxanthine and xanthine to form uric acid, releasing reactive oxygen species during this catalytic process [1-3]. Structurally, XO is a homodimer widely distributed in mammalian tissues, with a molecular mass of 290 kDa. Each subunit contains one molybdenum molybdopterin (Mo-pt), center in which the oxidation process occurs, one flavinadenine dinucleotide (FAD) and two distinct [2Fe–2S] centers [4-6].

This enzyme is responsible for the medical condition known as gout, characterized by hyperuricemia and causing oxidative damage to living tissues [4, 7]. Allopurinol is used for the treatment of this pathology, but it has been reported that prolonged use may cause serious side effects such as hepatitis, nephropathy, hypersensitivity and skin rash [7-9]. Thus, alternative XO inhibitors are needed. Aiming at this, numerous non-purine analogues [10] compounds such as, febuxostat [11], curcumin [12], naphthopyrans [13], aloe-emodin derivatives [14], pyrano[3,2-d]pyrimidine derivatives [15], hydroxychavicol analogs [16], flavonoids [1,4-6,8,17-19], chalcones [20] and flavonoid derivatives [21,22], were reported as XO inhibitors.

Previously, the influence of the substitution pattern of hydroxy groups on the inhibitory activity of flavones has been reported [1,5,8]. However, the influence of methoxy substituents has not been described.

Gardenia oudiepe Vieill. (synonym *G. cerifera* S.Moore) is a tree endemic to New Caledonia. The buds and young leaves of this species are covered with a yellow exudate containing rare polymethoxyflavones (PMF) [23]. This exudate constitutes a renewable raw material, its harvest does not damage the plant and

access to metabolites does not require any steps of time and solvent-consuming extraction. A single dissolution followed by paper filtration in order to remove small vegetal inclusions provides a mixture available for purification of target compounds.

According to the above presented, this work aimed to evaluate the XO inhibitory activity of PMF isolated and semisynthesized from *Gardenia oudiepe* and to establish the mode of ligand-enzyme interaction by molecular modeling.

These data may enable the design of new potent inhibitors of XO.

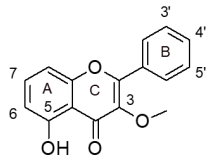
2. Results and discussion

2.1. In vitro inhibitory activity on xanthine oxidase

In this study seven flavones (compounds **1**-**6**, **9**; Fig. 1) isolated from *Gardenia oudiepe* and two semisynthetic derivatives (compounds **7** and **8**) obtained by methylation of **1** and **4** respectively, were evaluated for XO inhibitory activity.

All compounds showed a concentration-dependent inhibition. The IC_{50} values for **1-9** and the reference inhibitor Allopurinol were estimated using nonlinear fitting of concentration-response data (Table 1). Compounds **1**, **2** and **3** were active in the nanomolar range, being sixty-three, five and three times, respectively, more active than Allopurinol (IC_{50}, 0.25±0.004 µM) (Fig. 1).

Table 1. Structures and XO inhibitory activity of PMF.

Substitution pattern	Compound	6	7	3'	4'	5'	IC_{50} (μM)
	**1**	H	OH	H	OH	H	0.004±0.001*
	2	OCH_3	OH	H	OH	H	0.05±0.01*
	3	H	OH	OCH_3	OCH_3	OH	0.09±0.003*
	4	OCH_3	OH	H	OCH_3	H	0.22±0.08
	5	OCH_3	OH	OCH_3	OCH_3	OCH_3	4.54±0.10*
	6	OCH_3	OH	OCH_3	OCH_3	OH	6.95±1.30*
	7	H	OCH_3	H	OCH_3	H	9.79±1.40*
	8	OCH_3	OCH_3	H	OCH_3	H	14.90±1.20*
	9	H	OCH_3	OCH_3	OCH_3	OH	21.03±1.20*

Positive control: Allopurinol IC_{50} = (0.25±0.004) μM. Media ± SD of at least 3 determinations.*p<0.001, the values resulted significantly different to allopurinol.

2.2. Structure Activity Relationships

Previous reports highlighted some structural requirements as the presence of hydroxy groups in position 5, 7, and 4' of flavones [1,5,8]. The present work extends these data to compounds bearing a combination of hydroxy and methoxy substitutions, which were, to our knowledge, never evaluated for XO inhibitory activity.

All tested PMF share a core including a methoxy group on position 3, a hydroxy on position 5 and oxygenated aromatic carbons on positions 4' and 7. Thus, the minimal structure is identified as 3-methoxykaempferol (**1**). Variability is due to different substitution pattern on positions 6 and 7 of the A ring, and 3', 4', 5' of the B ring (Table 1).

Compound **1** has been identified as the most active PMF within this series, showing an IC_{50} of 4 nM. In comparison with kaempferol (IC_{50} 0.09±0.002 μM), methoxylation of the hydroxyl group in position 3 resulted in a stronger inhibitory effect. Additional methoxy group on position 6 (compound **2**) or modification on

the B ring (compound **3**) led to a 20 fold decrease of the inhibition. Nevertheless, **2** and **3** still demonstrated IC_{50} in the nanomolar range (Fig. 1).

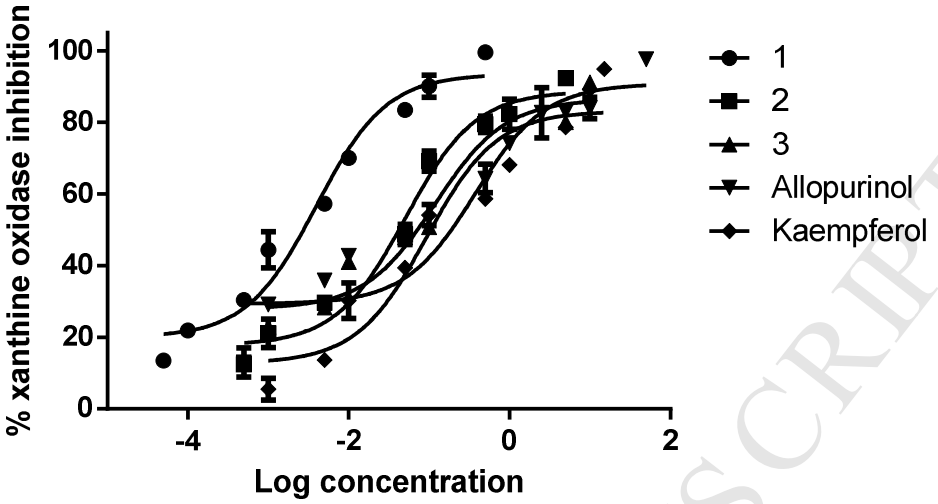


Fig. 1. Concentration-dependent inhibition of xanthine oxidase activity by compounds **1**, **2**, **3**, kaempferol and positive control allopurinol (N = 3).

Generally, the most active compounds possess only a 5,7-dihydroxy A-ring. PMF with a 5,7-dihydroxy-6-methoxy A-ring remain strong XO inhibitors if the B-ring has a low steric hindrance, such as **2** and **4**. With this type of A-ring, further substitution on the B-ring by bulkier groups is detrimental to the activity, as observed for **5** and **6**.

The replacement of hydroxy group with a methoxy group in position 7 also resulted in dramatically less active derivatives, exemplified by the IC_{50} of **8** and **9**, approximately 70 and 240 fold higher than those of **4** and **3**, respectively.

All this structural characteristics remain to be considered in the atomic level later in the docked XO-flavone complexes.

2.3. Docking studies

Computational docking studies were carried out using MOE™ tool (2014) [24], to understand the binding mode of the complexes formed between XO (PDB ID: 3NVY) [25] and the most actives flavones **1**, **2** and **3**.

XO is a homodimer composed by three chain each, with a tertiary structure of two domains, an alpha beta roll and a mainly alpha orthogonal bundle. The catalytic site is located in the first domain [26]. The residues contacting the ligand are near to a beta turn motifs, placed between the H1 alpha helix and a strand belonging to a beta sheet (Fig. 2). The co-crystallized ligand is in H-bond contact with mainly Arg880, Thr1010 and Glu802 [25] In the docking procedure (described in Methods Section) a sphere of 4.5 Å around the co-crystallized ligand was selected as "Site" for docking, including the amino acid residues above described as essential for catalysis.

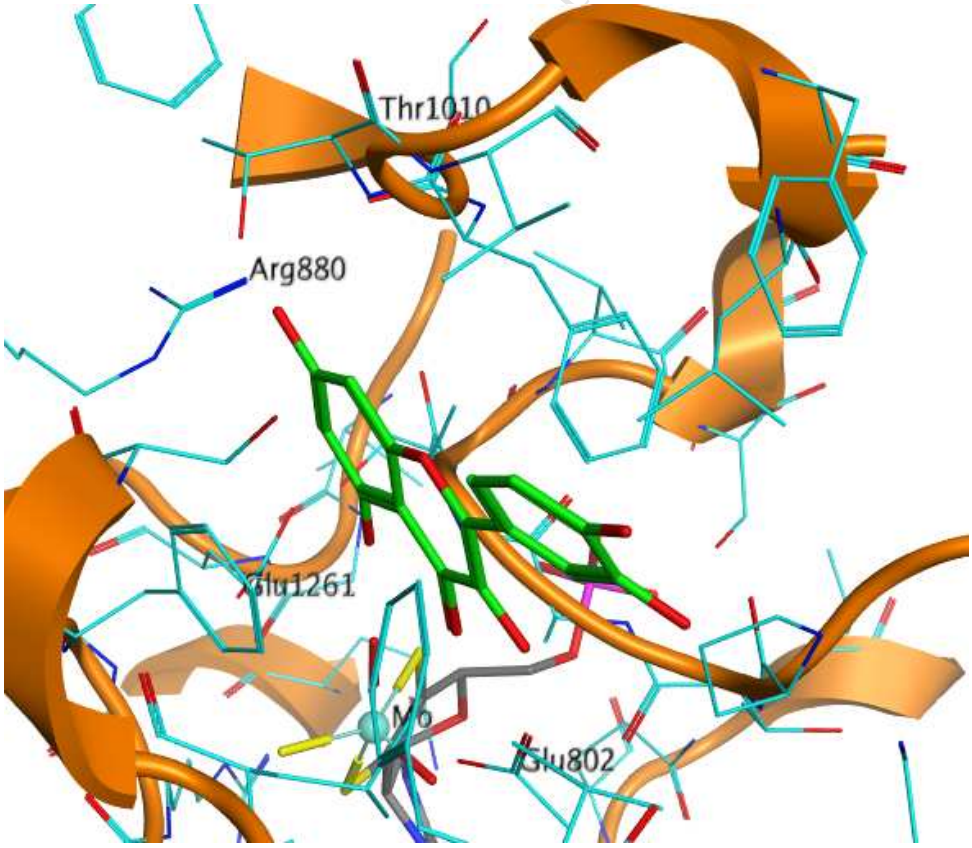
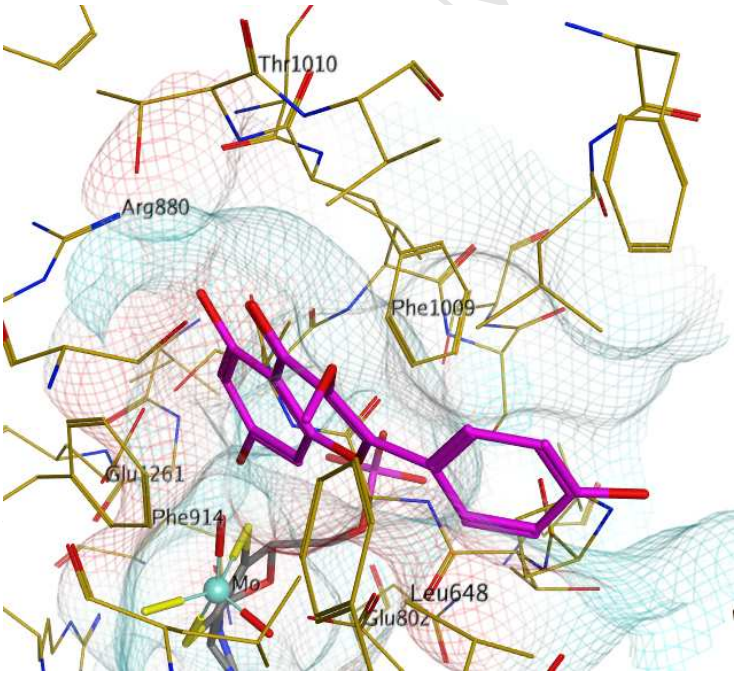


Fig. 2. Active site of xanthine oxidase (3NVY) complexed with quercetin.

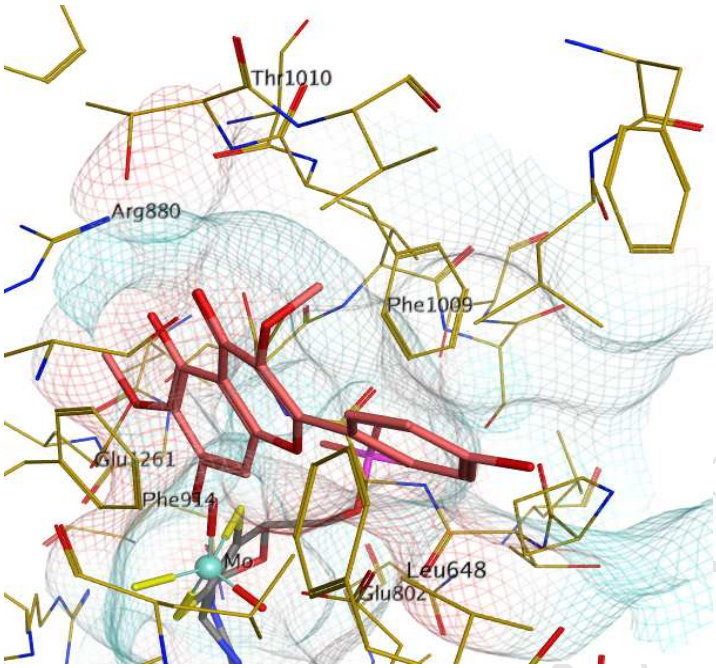
As we can observe in electrostatics 2D molecular surfaces (Fig. 3.a-c), the docking study showed that **1**, **2** and **3** were well located into the active site of XO, with the lowest binding energy of -8.2515, -7.7392 and -7.7166 kcal mol^{-1}, respectively. These results are in concordance with those obtained on *in vitro* assays (Table 1). All compounds were surrounded by several aminoacid residues (Glu802, Thr1010, Arg880, Phe914, Phe1009, Leu873, Val1011 and Leu648), that were described as catalytic residues [25]. Additionally, in Fig. 3 are shown the contacts that the compounds have established with the near residues.

For the less active flavones **4**-**9**, the ranking of the scores after docking was -8.3610, -8.5266, -8.2663, -8.0393, -8.1575, -7.8761, respectively.

3.a)



3.b)



3.c)

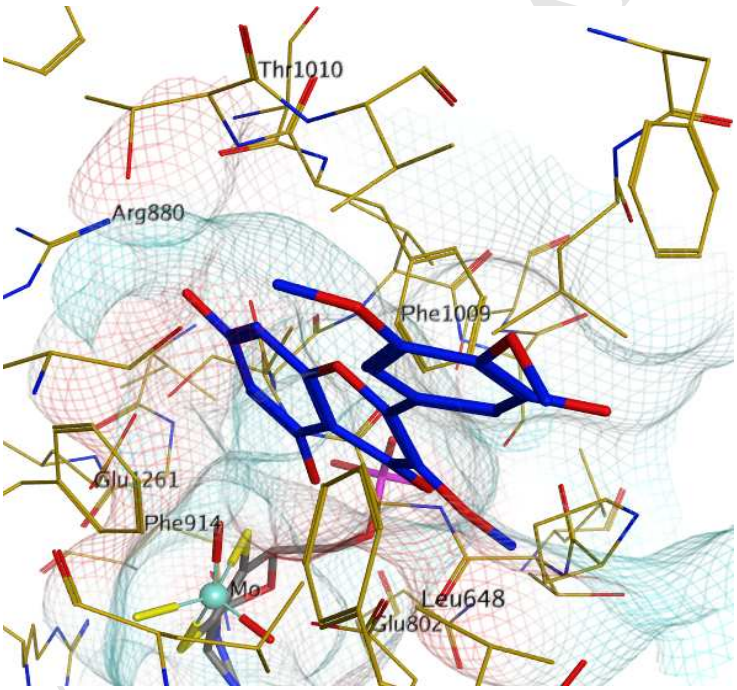


Fig. 3. a-c. 2D Electrostatic Surface Map of the best pose docked of **1**, **2** and **3** respectively. Surface map in the active site: in blue color are the electropositive surface areas and in red color are the negative electrostatic surfaces.

A 2D graphic description of the ligand interactions are shown in Fig. 4. The benzopyranone ring of flavones are sandwiched between Phe914 and Phe1009 residues, and for **1** and **2**, a π-π interaction between the A ring and the aromatic ring of the Phe914 residue was observed. This interaction was previously reported as important for the recognition of the ligand by XO [17]. It has been reported that Glu802, Glu1261 and Arg880 residues play key roles in the hydroxylation of substrate xanthine [25,27-29]. A hydrogen binding with Glu802 residue for all compounds was observed and a H-bond interaction between OH-7 and Glu1261, reinforcing the fact that OH-7 assists the inhibitory activity [29]. The B-ring exhibited a hydrophobic interaction with the Leu648 residue, which led to stabilization of the compound inside the active site [30]. Then, we could conjecture that those natural compounds would have the same binding region as the substrate of XO.

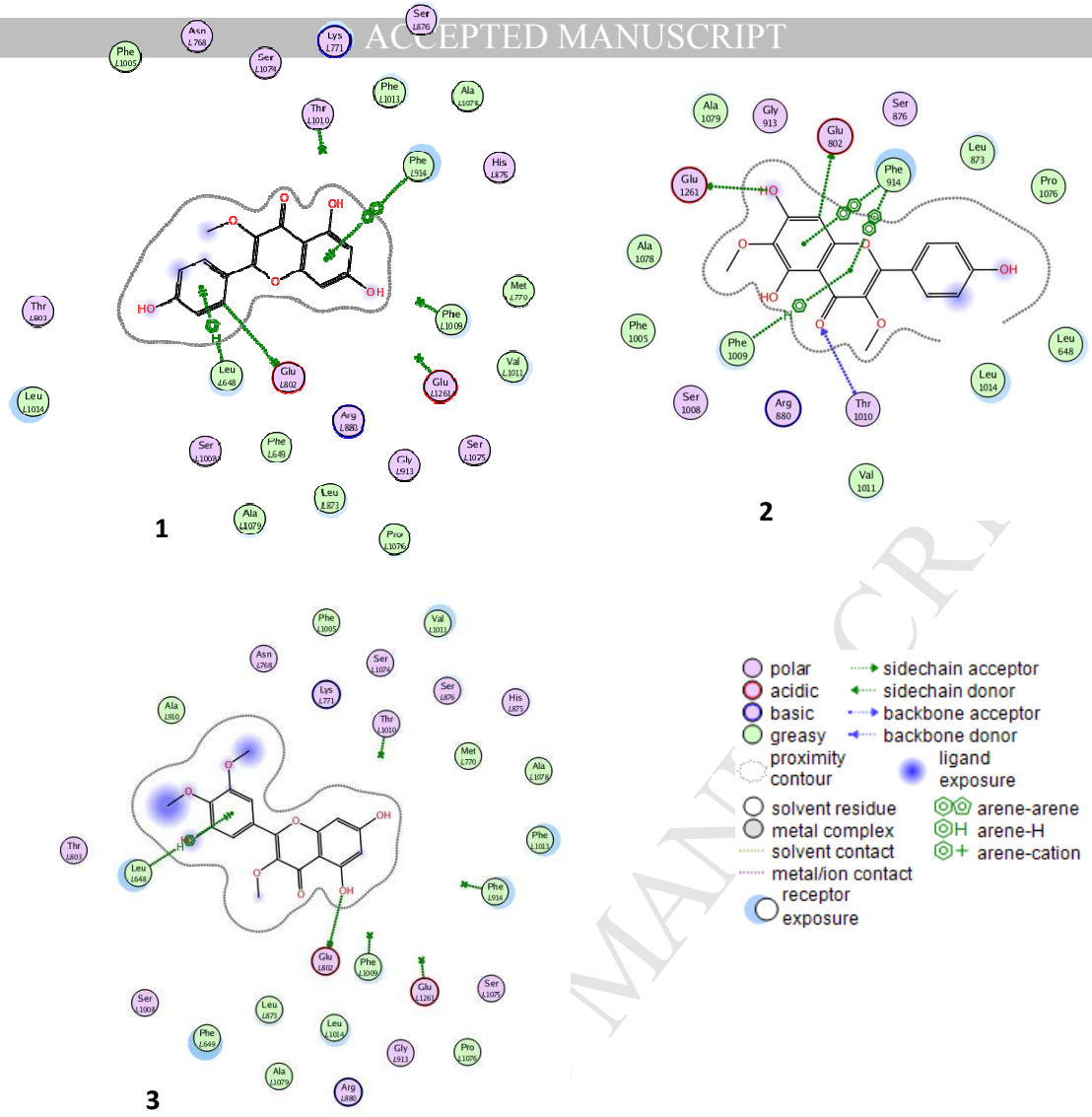


Fig. 4. Ligand interaction of **1**, **2** and **3** and residues of the active site of xanthine oxidase (3NVY).

Although some of the less active PMF such as **9** presented a good score ranking, interactions with crucial catalytic residues as Glu1261 was not observed. That fact supports the reported observation that the presence of Glu1261 is important to block the XO activity [25,28-30]. Another conclusion is that even if the docking procedure is a very useful tool to localize regions in the active site and visualize the preferred pose of studied molecules, it is not the best tool to measure interactions related to the IC_{50}. This observation could be explained by the recent

assertion that there is no obvious relationship between the affinities measured by this kind of formalism as docking and inhibition for flavonoids [5]. Further studies such as Molecular Dynamics or Steering molecule dynamic could be envisaged to complete the evaluation.

3. Conclusions

The xanthine oxidase inhibitory activity of nine natural or semisynthetic flavones was evaluated. The raw material is a renewable part of *Gardenia oudiepe*. Cultivation of this tree is easy. Thus, our work is compatible with some criteria of green chemistry and sustainable development. **1**, **2** and **3** were found to be the most active compounds with IC_{50} values ranging from 4 to 90 nM, being sixty-three, five and three fold more active respectively, comparing to allopurinol (0.25±0.004µM). **4** showed a similar activity to the reference inhibitor. Additionally, structure-activity relationships of these PMF were first established. The molecular docking of **1**-**3** showed important Van der Waals and hydrogen binding interactions with catalytic residues of the active site of XO as Leu648, Glu802, Glu1261, respectively, which could explain the potent inhibitory activity observed for those compounds. Additionally, the importance of 7-0H previously reported was confirmed, as well as the benzopyrane and the B-ring hydrophobic interactions with lipophilic residues that strongly contribute to the stabilization of the compounds in the active site.

These data suggest that active PMF inhibitors of XO may be regarded as candidates for the treatment of disorders where this enzyme is involved, as well as lead for further designing of new compounds with increased XO inhibitory potential.

4. Materials and Methods

4.1. Plant material

Bud exudates of *G. oudiepe* were collected in October 2008 in Forêt Plate, North Province of New Caledonia. A voucher specimen (POU-0290) was deposited at the Herbarium of the Botanical and Tropical Ecology Department of the IRD Center, Noumea, New Caledonia.

4.2. General procedure for the isolation of flavones 1-6 and 9

200.0 g of flowering buds and leaf bases of *G. oudiepe* covered with exudate was dissolved in 1L of dichloromethane. The solution was filtered using a Buchner funnel. After evaporation to dryness under reduced pressure, 52.0 g of exudate free from buds and leaf pieces were recovered. Repeated chromatographic separations on silica gel column using as eluent gradients of increasing polarity cyclohexane/dichloromethane and dichloromethane/methanol provided compounds 1-6 and 9. Flavones 1-2 and 4-6 were identified by comparison with recently published ^{1}H and ^{13}C NMR data [23, Supporting Information]. Compound 3: 3,3',4'-trimethoxy-5,5',7-trihydroxyflavone; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 12.63 (bs, 1H, OH-5), 7.27 (d, 1H, *J*=2 Hz, H-6'), 7.22 (d, 1H, *J*=2 Hz, H-2'), 6.48 (d, 1H, *J*=2 Hz, H-8), 6.24 (d, 1H, *J*=2 Hz, H-6), 3.89 (s, 3H, OCH_3-3'), 3.85 (s, 3H, OCH_3-3), 3.80 (s, 3H, OCH_3-4'); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 178.9 (C-4), 165.3 (C-7), 162.2 (C-5), 157.3 (C-9), 155.9 (C-2), 153.9 (C-3'), 151.5 (C-5'), 139.8 (C-4'), 139.4 (C-3), 126.9 (C-1'), 110.7 (C-6'), 105.2 (C-10), 104.7 (C-2), 99.6 (C-6), 94.7 (C-8), 60.9 (OCH_3-4'), 60.8 (OCH_3-3), 56.8 (OCH_3-3'). Compound 9: 3,3',4',7-tetramethoxy-5,5'-dihydroxyflavone; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 12.57 (bs, 1H, OH-5), 7.35 (d, 1H, *J*=2 Hz, H-6'), 7.33 (d, 1H, *J*=2 Hz, H-2'), 6.44 (d, 1H, *J*=2 Hz, H-8), 6.35 (d, 1H, *J*=2 Hz, H-6), 6.03 (bs, 1H, OH-5'), 4.01 (s, 3H, OCH_3-4'), 3.93 (s, 3H, OCH_3-3'), 3.88 (s, 3H, OCH_3-3),

3.87 (s, 3H, OCH_3-7); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 178.8 (C-4), 165.6 (C-7), 162.0 (C-5), 156.7 (C-9), 155.4 (C-2), 152.1 (C-3'), 149.2 (C-5'), 139.5 (C-3), 137.8 (C-4'), 125.9 (C-1'), 108.6 (C-6'), 106.1 (C-10), 105.0 (C-2), 98.0 (C-6), 92.2 (C-8), 61.1 (OCH_3-4'), 60.3 (OCH_3-3), 56.1 (OCH_3-3'), 55.8 (OCH_3-7).

4.3. General procedure for the semi-synthesis of flavones 7 and 8

Methylation of compounds 1 and 4 gave respectively flavones 7 and 8 (yield 69% and 71%). Reactions were performed by stirring at room temperature the substrate (100 mg) with dimethyl sulfate (Me_2SO_4, 4 equiv.) and 1,8-diazabicyclo[5.4.0]undec-7-en (DBU, 2 equiv.) in dried acetone for 1 h. The crudes were precipitated and washed with iced water. The resulting residues were solubilized with ethyl acetate (15 mL) and treated with a solution of 1N HCl (3 mL). The final products were extracted with ethyl acetate (3 to 10 mL); the organic phases were washed with a saturated solution of NaCl and dried over Na_2SO_4. After filtration and solvent evaporation, methylated compounds were purified by chromatography on a silica gel column, using as eluent a mixture of dichloromethane/methanol (95/5, v/v). ^1H and ^{13}C NMR chemical shifts of derivatives 7 and 8 were in agreement with literature data [23, Supporting Information].

4.4. In vitro xanthine oxidase inhibitory activity

The assay was performed as previously described by Schmeda-Hirschmann et al., 1992 [31]. Briefly, the assay medium consisting of 21.4 µL of xanthine oxidase solution from bovine milk (0.04 U/mL, Sigma Chemical Co., St Louis, MO, USA), and 0.75 mL of the control solution [K_2HPO_4/ KH_2PO_4 buffer (0.07 M, pH 7.5)] or the sample solution [prepared with each compound dissolved in DMSO and subsequently diluted to the appropriate concentrations with the above

buffer] were mixed and preincubated at 25 ℃ for 15 min. Then, 0.45 mL of the xanthine solution substrate (150 µM, Sigma Chemical Co., St Louis, MO, USA) was added and preincubated at 25 ℃ for 30 min. The reaction was then stopped by addition of 1 mL of HCL (1N) and the absorbance was measured at 290 nm on a Cary Win UV-VIS spectrophotometer, Varian, Inc., Agilent Technologies (Santa Clara, USA). Allopurinol (Sigma Chemical Co., St Louis, MO, USA) was used as positive inhibitor control. Each treatment was replicated three times. The percent inhibition of xanthine oxidase activity was calculated as follows: % inhibition = $[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$, where $Abs_{control}$ is the absorbance of the control solution and Abs_{sample} is the absorbance of the sample solution.

4.5. Calculations and statistics:

All assays were independently performed in triplicate, and results were expressed as media ± SD of three separate experiments. The IC_{50} values were estimated using the *GraphPad Prism 6.0* software on a compatible computer. The results were analyzed by unidirectional analysis of variance (ANOVA) followed by the *Bonferroni's* test for multiple comparisons using *GraphPad Prism 6.0* software.

4.6. Molecular docking studies:

The crystal from milk bovine xanthine oxidase (PDB ID: 3NVY), co-crystallized with quercetin at 2.0 Å resolution was obtained from the Protein Data Bank (http://www.rcsb.org/pdb) [26]. All water molecules were deleted and the hydrogens atoms and charges were adjusted with the MMFF94x forcefield from MOE™ suite (Chemical Computing Group Inc., http://www.chemcomp.com) [32,33]. The 3D structures were built and minimized in MOE, using the same forcefield above mentioned. A conformational search with LowmodeMD was

carried out to generate different conformers for the docking. LowmodeMD is a conformations search method that uses a short ~1 ps run of Molecular Dynamics (MD) at constant temperature followed by an all-atom energy minimization of all compounds to generate conformations [34]. The docking was performed considering all residues within a 4.5 Å sphere centered on quercetin atoms. As a placement function was selected Alpha Triangle and the scores were calculated with the Affinity ΔG function, which measures the enthalpic contribution to the free energy of binding (MOE^{TM} Chemical Computing Group, 2009), in concordance with a validation procedure [35,36] to reproduce by docking, the same pose of quercetin in the crystal structure. The graphical representations of the calculated binding poses were performed by Surface Maps, and Ligand Interaction MOE^{TM} tools.

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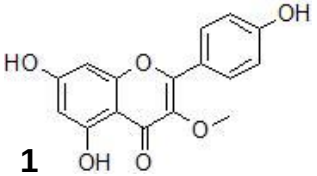
***Gardenia oudiepe*'s exudate**



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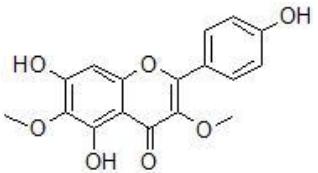

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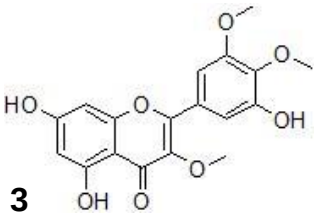

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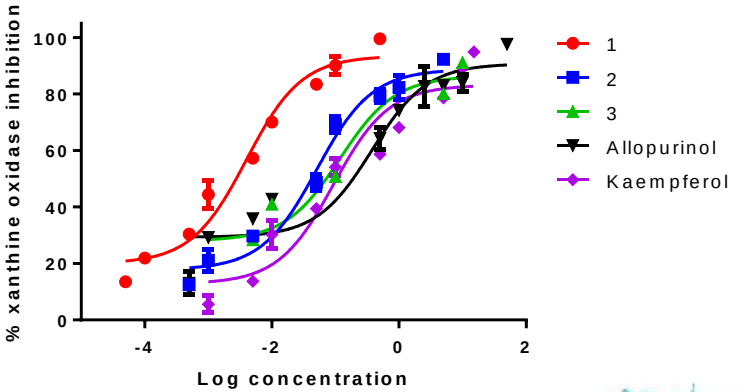

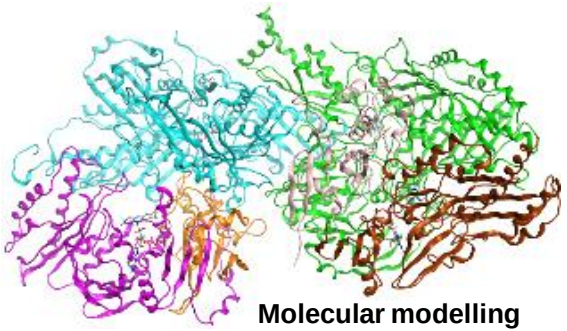

Molecular modelling

Highlights

- Xanthine oxidase inhibitory activity of polymethoxyflavones from *Gardenia oudiepe* was determinate.
- Some compounds were more actives than Allopurinol, with IC_{50} in the nanomolar range.
- SAR and docking studies were determinate for those compounds for the first time.