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Primary Virtual and *in vitro* Bioassay Screening of Natural Inhibitors from Flavonoids against COX-2

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[ABSTRACT] In this study, we reported the screening of 9 compounds of flavonoids from the ZINC and PubChem databases (containing 2 092 flavonoids) using the iGEMDOCK software tool against the COX-2 3D protein structures. Each compound was also evaluated by an *in vitro* bioassay testing the inhibition of COX-2. Centaureidin and luteolin were found to be the potential inhibitors of COX-2 as demonstrated by IC_{50}: 45 and 36.6 μmol·L^{-1}, respectively. In addition, structure activity relationships and other important factors of the flavonoids binding to the active site of COX-2 were discussed, which is expected for further rational drug design.
[KEY WORDS] Inhibitor; Flavonoids; COX-2; Structure activity relationship; Bioassay

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1 Introduction

Natural products have long been recognized as an important source of therapeutically effective agents [1]. Flavonoids are a group of naturally occurring polyphenols that are ubiquitously distributed in various foods and beverages of plant origin. These compounds display many bioactive and therapeutic properties [2-4]. COX-2 is a key enzyme for the production of prostaglandins (PGs) which frequently cause inflammation and pain when over expressed. Inhibitors of COX-2 work by inhibiting the production of PGs, fatty-acid derivatives well-known for their anti-inflammatory effects in arthritis treatment [5].

Virtual screening of chemical databases is now a well-established method for finding new drug leads provided that a three-dimensional structure of the target is known. As the number of pharmaceutical targets is predicted to dramatically increase in the coming years [6], virtual screening methods have undoubtedly played a major role in pharmacology by finding the very first leads of new targets. Collecting all the available natural compounds and screening them randomly target by target is laborious, because it is costly and also time consuming in isolating and screening compounds. Virtual screening has the potential to solve this problem. Virtual screening by docking shows great promise in active compound discovery. It was demonstrated that virtual screening enriched the hit rate by thousands-fold over random screening [7].

The main goal of this work was to obtain COX-2 inhibitors from flavonoid compounds by virtual screening and bioassay, and summarize the structure-activity relationship between flavonoids and COX-2.

2 Materials and Methods

2.1 *Reagents*

Myricetin and isoquercitin were purchased from Sigma Chemical (China mainland). Apigenin, quercetin, luteolin, scutellarein, genistein and rutin were purchased from Nanjing TCM Institute of Chinese Materia Medica. Centaureidin was purchased from CFMOT-PHYTO (Germany). COX Fluores-

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cent inhibitor screening assay kit was obtained from Cayman Chemical Company (Ann Arbor, Michigan, USA).

2.2 *Structures of COX-2 and its substrates*

The favorable binding orientations of the competitive inhibitor of COX-2 were focused on the active site. The 3D crystal structure of COX-2 in complex with arachidonic acid (AA) (PDB code: 1CVU) was used as a basis for COX-2 docking. The 3D structures of the ligands, including flavones, flavonols, isoflavones, flavone glycosides, chalcones and flavanones, were downloaded from online compound databases, such as ZINC (http://zinc.docking.org/) and PubChem in the SDF file format. The SDF files were then separated into MOL files using openbabel (http://openbabel.org/wiki/install). A ligand database was composed of these structures, which contained 2 092 Lipinski Rule of 5 compliant molecules, having molecular weights in the range 150 to 500, calculated AlogP98 < 5, number of hydrogenbond donors ⩽ 5, and number of hydrogen-bond acceptors ⩽ 10.

2.3 *iGEMDOCK software*

Model building, ligand docking, and energy minimization of the complexes were all performed by using iGEMDOCK, which is developed by the University of National Chiao Tung, Taiwan (http://gemdock.life.nctu.edu.tw/dock/) [8]. iGEMDOCK is a graphical-automatic drug discovery system, for integrating docking, screening, post-analysis, and visualization. Using iGEMDOCK, the predicted ligand binding geometries generated are able to be directly visualized by a molecular visualization tool and analyzed by post-analysis tools. An empirical scoring function and evolutionary approaches are adopted in iGEMDOCK. The scoring function of iGEMDOCK is composed of different terms that address the energy contributions from electrostatic, steric, and hydrogen bonding potentials. For steric and hydrogen bonding potentials, a linear model was employed, which is simple but efficient in recognizing potential binding orientations. The electrostatic energy was calculated by formal charges and converted into kilocalories per mole [9].

2.4 *Virtual screening*

First, iGEMDOCK specified the coordinates of substrate and protein atoms, and selected the atoms that comprised the substrate binding area. Then, the center of the binding site of the receptor and the search cube of the binding site were automatically determined according to the maximum and minimum of coordinates of these selected protein atoms. iGEMDOCK computes a ligand conformation and orientation relative to the binding site of target receptor by using a generic evolutionary method (GEM). After the ligands and protein were prepared, we set the size of binding site (8 Å) and the parameters such as initial step sizes ($\sigma = 0.8$, $\Psi = 0.2$ (in radius)), family competition length ($L = 2$), population size ($n = 300$), recombination probability ($pc = 0.3$), generations (80), and number of solutions ($n = 10$) to values typically employed in a standard docking run.

All compounds that scored better (i.e. lower) than the native substrate were further screened for inhibitory potential against COX-2.

2.5 *COX-2 inhibitory activity in vitro*

Cayman's COX Fluorescent inhibitor screening assay provides a convenient fluorescent based method for screening human recombinant COX-2 for isozyme specific inhibitors. The assay utilized the peroxidase component of COXs. The reaction between PGG2 and AFHP (10-acetyl-3, 7-dihydroxyphenoxazine) produced the highly fluorescent compound resorufin. Resorufin fluorescence can be easily analyzed with an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm. The COX-2 assay consisted of a 200 μL reaction mixture containing 150 μL assay buffer, 10 μL Heme, 10 μL COX-2, 10 μL fluorometric substrate, and 10 μL test solution (20, 50, 100, 200 $\mu mol \cdot L^{-1}$) The reactions were initiated by quickly adding 10 μL AA, then incubating for 2 min at room temperature. Dup-697 was used as a positive control. IC_{50} values were calculated from the mean values ($n = 3$).

2.6 *Statistics*

All data are expressed as $\bar{x} \pm s$ of triplicate experiments. Statistical differences of the potency of the inhibitors were tested for significance using the Student's *t*-test at a level of $P < 0.05$ (SPSS v. 16)

3 Results

3.1 *Virtual screening of flavonoids*

Before screening the database we constructed, the docking protocol was validated. The known COX inhibitor AA was docked into the binding pocket to obtain the docked pose (Fig 1) and the RMSD (Root Mean Square Deviation) of all

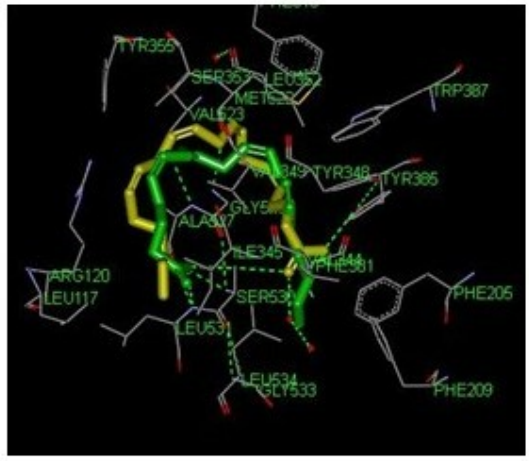


Fig. 1 docked AA (green part) occupies the COX-2 substrate binding site, the yellow molecule is the substrate. AA is bound in an pocket formed by Tyr385, Ser530, Val523, Arg120, Tyr387, Met522, Ser353, Phe518, Leu534, Arg527, Ile345 and Gly520. The parameters for the docking simulation are good in reproducing the *X*-ray crystal structures ligand confirmations. Fig. 1 was drawn by Discovery studio 2.5.0.

atoms between these conformations is 1.555 Å (calculated by SPDBV.4.01) indicating that the parameters for docking simulation are good enough to reproduce the *X*-ray crystal experimental results. AA is bound in a pocket formed by Tyr385, Ser530, Val523, Arg120, Tyr387, Met522, Ser353, Phe518, Leu534, Arg527, Ile345, Gly520. The number of flavonoids which could interact with COX-2 was 9 according to the scores lower than the substrat: AA (Table 1).

3.2 *Competitive inhibition of COX-2 from flavoniods*

2 flavonoids demonstrated COX-2 inhibiting activity (Table 2, Fig. 2), they are centaureidin and luteolin (IC_{50}: 46.7, 57.2 μmol·L^{-1}), centaureidin showed an inhibitor greater than 50% at 50 μmol·L^{-1}. As the rough IC_{50} had been got, we set a few concentrations of centaureidin and luteolin around 46.7/57.2 μmol·L^{-1} (35, 45, 55, 65 μmol·L^{-1}/40, 50, 60, 70 μmol·L^{-1}) and do the bioassay to obtain an accurate IC_{50}. Finally, the more accurate IC_{50} of centaureidin/luteolin is 45 μmol·L^{-1}/ 36.6 μmol·L^{-1} (Figs. 3 and 4).

3.3 *SAR between flavonoids and COX-2*

We can summarize the structure-activity relationship between flavonoids and COX-2 as follows. Flavononones and chalcones do not have an inhibitory effect on the COX-2 protein. Within flavones, the olefin at C2 and C3 forms a planar structure which is important foe inhibiting COX-2. 5,

Table 1 Chemical structures and scores of the various flavonoids tested for the inhibitors of COX-2 by virtual screening

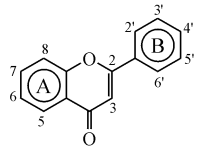
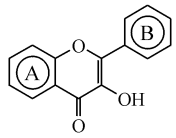
Chemical formula	Name	Score of COX-2	Substitution							
			5	6	7	8	2’	3’	4’	5’
Flavone	Flavone	−86.091 8								
	Luteolin	−101.637	OH	H	OH	H	H	OH	OH	H
	Apigenin	−108.119	OH	H	OH	H	H	OH	OH	OH
	Scutellarein	−105.053	OH	OH	OH	H	H	OH	H	H
Flavonol	Quercetin	−107.628	OH	H	OH	H	H	OH	OH	H
	Myricetin	−114.759	OH	H	OH	H	H	OH	OH	OH
	Genistein	−132.135	OH	OMe	OH	H	H	OH	OMe	H
	Centaureidin	−124.000	OH	OMe	OH	H	H	OMe	OH	H
Flavone glycoside	Isoquercitrin	−135.034	OH	H	OH	H	H	OH	OH	H
	Rutin	−126.638	OH	H	OH	H	H	OH	OH	H
original ligands (COX-2)	AA	−96.605 3								

Table 2 Inhibition ratio and IC_{50} of potential COX-2 inhibitors

Name	Inhibition ratio/%				IC_{50}/μmol·L^{-1}
	20 μmol·L^{-1}	50 μmol·L^{-1}	100 μmol·L^{-1}	200 μmol·L^{-1}	
DUP-697	42.8 ± 0.001	66.7 ± 0.001	80.2 ± 0.002	94.0 ± 0.001	24.3
Centaureidin	36.0 ± 0.003	52.0 ± 0.003	54.9 ± 0.001	69.9 ± 0.001	46.7
Luteolin	52.0 ± 0.003	54.9 ± 0.001	60.1 ± 0.001	69.9 ± 0.001	57.2
Myricetin	9.9 ± 0.001	19.8 ± 0.001	36.0 ± 0.003	69.9 ± 0.008	> 200
Quercetin	43.3 ± 0.004	43.4 ± 0.005	43.7 ± 0.001	44.3 ± 0.001	> 200
Genistein	—	—	3.4 ± 0.001	5.8 ± 0.003	> 200
Apigenin	—	—	—	—	—
Scutellarein	—	—	—	—	—
Isoquercitrin	—	—	—	—	—
Rutin	—	—	—	—	—

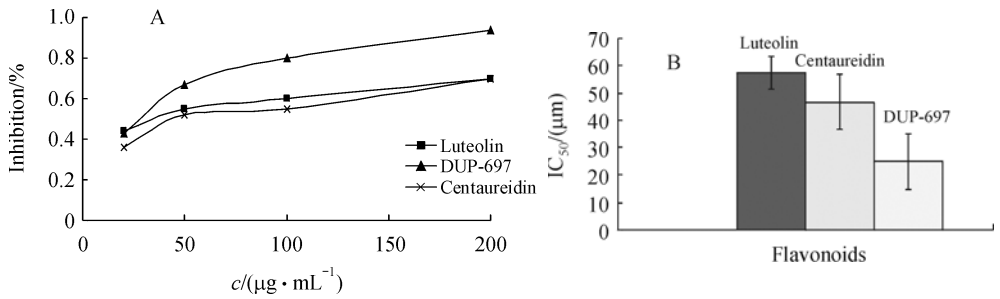


Fig. 2 A, inhibition ratio (%) of COX-2 by flavones. B, the calculated IC_{50} of the flavones for inhibition of COX-2, 2 flavonoids demonstrated COX-2 inhibiting activity, one of which (centaureidin) showed an inhibition greater than 50% at 50 μmol·L^{-1}. DUP-697 is a positive control

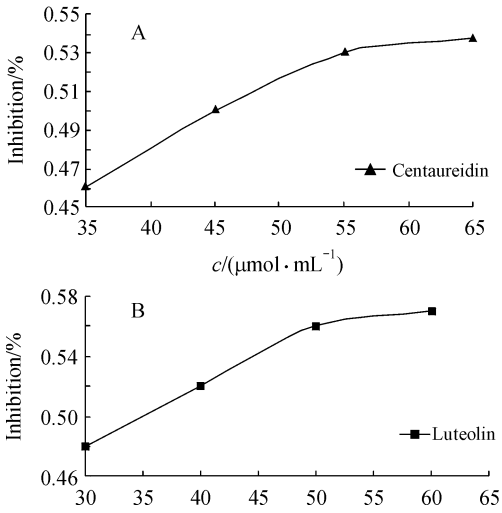


Fig. 3 A, more accurate inhibition ratio (%) of COX-2 by centaureidin. B, more accurate inhibition ratio (%) of COX-2 by luteolin

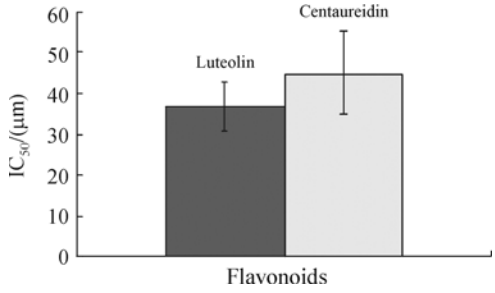


Fig. 4 The more accurate IC_{50} of the centaureidin and Luteolin for inhibition of COX-2

7-, 6, 7-, 7, 8-Dihydroxyl is ineffective, but inhibitory action could be exhibited if a hydroxyl is contained in the B ring, such as in luteolin, where the 3'-OH coordinates with Ser353 and the 4'-OH coordinates with Leu352 (Fig. 5A). Within flavonols, the olefin at C2 and C3 forms a plain，which is very important too. 5, 7-Dihydroxyl enhances the inhibitory effect on COX-2, and the substituent moiety at C3' and C4' also strengthen the inhibitory effects on COX-2 as does the carbonyl group at C4. In quercetin, the C7 hydroxyl is bound with Ile517, Phe518 and Gln192, the C5 hydroxyl interacts with Ser 355 and Leu352, the C4 carbonyl group is close to Tyr355, and the C3' hydroxyl interacts with Tyr385 (Fig. 5B). A B ring capable of rotating is important too, as morin has no effect in inhibiting the COX-2 protein due to the absence of rotation of the B ring under the action of a 2'-OH. The glycosyl substitution on C3 has minimal effect on the binding of inhibitors and COX-2. These results provided the basis for the interaction of flavonoids with COX-2, which may be helpful for the rational design of new potential drugs for COX-2 inhibition. Moreover, it also accounts for the molecular basis for the prevention of inflammation of various natural products containing flavonoids.

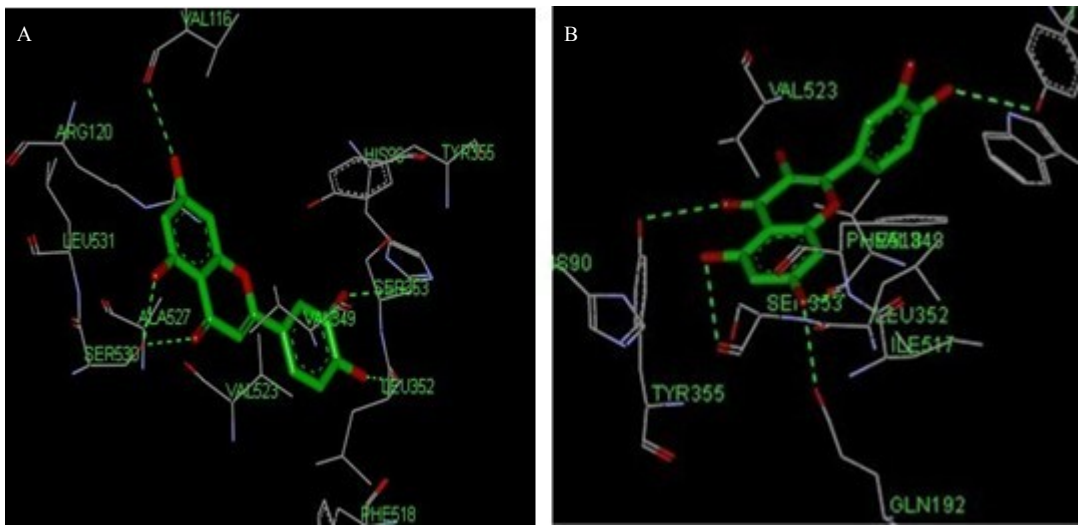


Fig. 5 A, molecular model of luteolin (green) binding in the active site of COX-2 with the 3'-OH coordinating with Ser353 and the 4'-OH coordinating with Leu352; B, molecular model of quercetin (green) binding in the active site with the C7 hydroxyl bound with Ile517, Phe518 and Gln192, the C5 hydroxyl interaction with Ser 355 and Leu352, the C4 carbonyl group close to Tyr355, and the C3' hydroxyl interacting with Tyr385. Fig. 5 was drawn by Discovery studio 2.5.0.

4 Discussion

The 9 flavonoids in Fig. 6 showed better ability of binding to the COX-2 substrates binding site by the scores come from virtural screening, which was comparable with AA. Luteolin, apigenin and scutellarein are flavones, quercetin, myricetin and centaureidin are flavonols, genistein is isoflavones, isoquercitrin and rutin are flavone glycoside, there are no isoflavones, chalcones and flavanones. Quercetin and isoquercetin are *Toona sinensis*, therefore, this plant maybe have the ability of resistance to COX-2.

Some of the molecules suggested by docking to be good inhibitors were not experimentally found to be as good as the docking suggested. It is likely to be related to either the scor-

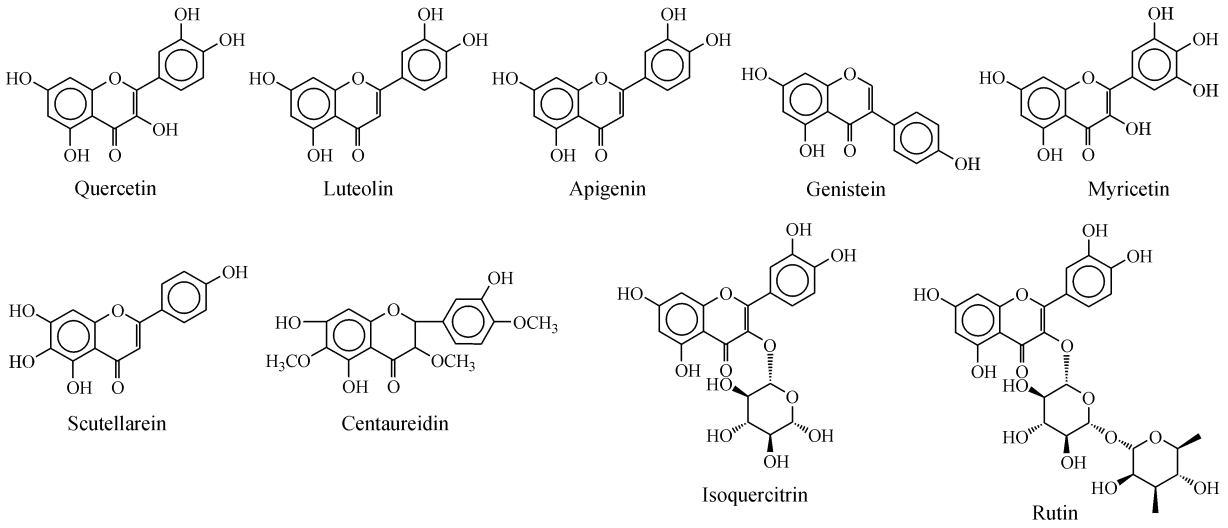


Fig. 3 Structures of flavones and isoflavonoids

ing function or how it computes its flexible ligand conformations. In the field of computer-aided drug design, a compound can be properly twisted to suit the structure of its receptor, however, it can not do this *in vivo* and *in vitro* therefore, *in vitro* experiment, only part of molecular conformation occupy the binding site of receptors.

Abbreviation

COX-2: cyclooxygenase-2; PGs: prostaglandins; AA: arachidonic acid; GEM: generic evolutionary method; PGG2: prostaglandin-G2; AFHP: 10-acetyl-3, 7-dihydroxyphenoxazine; RMSD: Root Mean Square Deviation

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从黄酮类化合物中初步虚拟筛选天然产物类 COX-2 抑制剂及其体外生物活性检测

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【摘　要】 **目的**：从黄酮类化合物中筛选 COX-2 抑制剂。**方法**：通过虚拟筛选及体外生物活性检测进行筛选。**结果**：通过 iGEMDOCK 软件从 ZINC 以及 PUBCHEM 数据库中筛选得到 9 个具有 COX-2 抑制作用的黄酮类化合物, 每个化合物进行了体外活力检测并计算 IC_{50}, 其中矢车菊黄素和木犀草素的 IC_{50} 分别为 45 和 36.6 μmol·L^{-1}。**结论**：矢车菊黄素和木犀草素可能是具有潜在 COX-2 抑制作用的化合物。

【关键词】 抑制剂; 黄酮类化合物; COX-2; 构效关系; 生物活性

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