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In vitro COX-1 and COX-2 enzyme inhibitory activities of iridoids from *Penstemon barbatus*, *Castilleja tenuiflora*, *Cresentia alata* and *Vitex mollis*

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ABSTRACT

A group of sixteen iridoids isolated from plants used as anti-inflammatory remedies in Mexican folk medicine were evaluated for their potential to inhibit cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) enzymes. From these assays, loganic acid (**10**) was identified as the most promising compound with both COX-1 (36.0 ± 0.6%) and COX-2 (80.8 ± 4.0%) inhibition at 10 μM. Compound **10** shows a better inhibition against the COX-2 enzyme. Other iridoids tested in the present study showed weak or no inhibition against these enzymes. Furthermore, herein are presented key interactions of iridoid **10** with COX-1 and COX-2 enzymes through molecular docking studies. These studies suggest that **10** exhibits anti-inflammatory activity due to COX inhibition.

Inflammation is present in several disorders and diseases like atherosclerosis, diabetes and cancer.[1–3] Thus, anti-inflammatories represent one of the most used groups of compounds to treat many clinical conditions.[3–5] Historically, people were dependent on folkloric medicine to treat these complaints. Hence, the research on plant species and its secondary metabolites is a promising tool.[6,7] In this regard, iridoids represent a class of novel natural products with anti-inflammatory activity that have been purified by bioassay-guided studies.[8] Iridoids are bicyclic monoterpenes containing a pyran ring in their structure. They can include a sugar moiety, typically β-glucose, linked through β-hemiacetal bond to C1. Families rich in this type of monoterpene are Apocynaceae, Lamiaceae, Loganiaceae, Rubiaceae, Scrophulariaceae and Verbenaceae.[8]

Cyclooxygenase-1 (COX-1) is an enzyme expressed constitutively in almost all cells and has great ubiquity in colon, kidney, spleen, stomach, liver, lungs, heart and brain. This explains the broad spectrum of biological responses and its cytoprotective properties, mainly in the gastrointestinal tract. In contrast, Cyclooxygenase-2 (COX-2) is not expressed constitutively in cells, but can be induced by cytokines, growth factors and endotoxins.[9,10] Both COX enzymes produce prostaglandins (PGs) which are involved in vascular, pain and inflammatory processes. By acting on arachidonic acid they produce PGG2 that is converted into PGH2. This PGH2 is transformed to one of five final metabolites, PGE2, PGD2, PGF2α, PGI2 or TxA2, depending on the tissue.[10] Therefore, it is suggested that COX-2 is the target for anti-inflammatory responses.

In the present study, sixteen iridoids isolated from four species (*Penstemon barbatus* Kunth, *Castilleja tenuiflora* Kunth, *Cresentia alata* Kunth and *Vitex mollis* Kunth) were evaluated for their inhibitory activity against COX-1 and COX-2 (Table S1), with the purpose of establishing their potential as anti-inflammatory agents.

Aerial parts of *Penstemon barbatus*, the whole plants of *Castilleja tenuiflora*, the leaves of *Cresentia alata* (all three species belonging to Bignoniaceae) and the leaves of *Vitex mollis* (Verbenaeae) were collected in different localities from Morelos state, Mexico: Cuernavaca, Huitzilac, Sierra de Huautla and Tepalcingo, respectively. A voucher specimen for each plant was deposited at Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos, México in two herbariums. Firstly, at CIBYC Herbarium with voucher numbers Bonilla-585 (*P. barbatus*) and Bonilla-616 (*C. tenuiflora*) and at HUMO Herbarium with voucher numbers 14111 (*C. alata*) and 19052 (*V. mollis*).

Compounds were isolated by open column chromatography (CC). The isolation procedures and purity of compounds were

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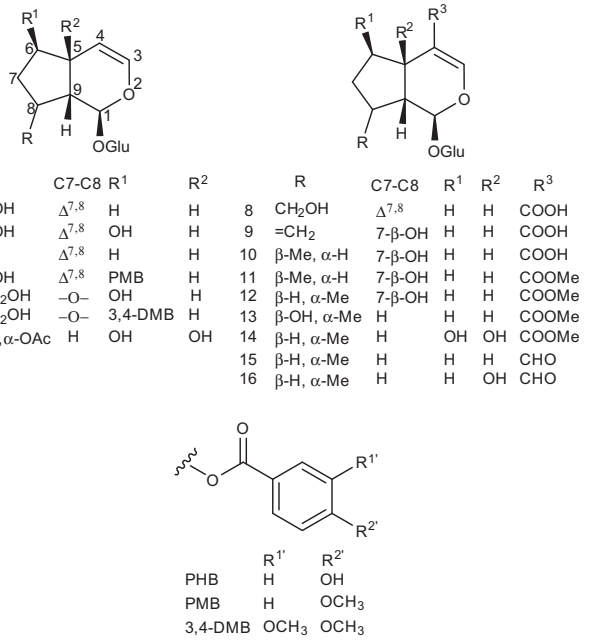
monitored by thin layer chromatography (TLC; precoated Kieselgel 60 F_{254} plates, 0.25 mm thick), and were visualized with UV light or by spraying them with anisaldehyde solutions followed by heating. The identity of all compounds was determined by ^{13}C NMR data (NMR spectra were recorded on Varian Unity 500 and 400 NMR spectrometers at 25 °C, using CD_3OD or D_2O as solvent),[11] which were compared with those reported in literature. Chemical shift were referenced to tetramethylsilane (TMS) as an internal standard.

The air-dried plant material was ground and extracted by exhaustive maceration at room temperature with *n*-hexane, dichloromethane and methanol (3 × 72 h). Extracts were recovered by rotary evaporation under vacuum. Extraction of 684 g of *P. barbatus*, 238 g of *C. tenuiflora*, 289 g of *C. alata* and 869 g of *V. mollis* yielded 143.3 g (20.95%), 21.4 g (8.99%), 23.9 g (8.26%) and 34.8 g (4.00%) of MeOH extracts, respectively. MeOH extracts were subjected to CC over reversed-phase in a Merck Lobar (RP-18) column for *P. barbatus* and *C. tenuiflora*, and over normal phase on Silicagel 230–400 mesh for *C. alata* and *V. mollis*. On these bases of TLC, fractions obtained were pooled in groups that were separated in pure compounds by successive chromatography column.

The MeOH extract from *Penstemon barbatus* (5.4 g) was dissolved in H_2O and fractionated by vacuum liquid chromatography (VLC, 80 mL fractions) over RP C-18 silica gel using H_2O/MeOH mixtures to yield geniposidic acid (**8**, H_2O/MeOH 5:1; 53 mg, 0.98%),[12] gardoside (**9**, H_2O/MeOH 5:1; 17 mg; 0.029%),[13] boschnaloside (**15**, H_2O/MeOH 4:1; 87 mg, 1.61%),[14] and plantarenaloside (**16**, H_2O/MeOH 4:1; 55 mg, 1.01%)[15] Figure 1.

The MeOH extract from *Castilleja tenuiflora* (6.0 g) was dissolved in H_2O and fractionated by VLC (100 mL fractions) over RP C-18 silica gel eluting with H_2O/MeOH mixtures to yield geniposidic acid (**8**, H_2O/MeOH 10:0; 208 mg; 3.46%;), aucubin (**2**, H_2O/MeOH 15:1; 86 mg; 1.34%),[12] bartioside (**1**, H_2O/MeOH 3:1; 89 mg; 1.48%),[15] 8-*epi*-loganin (**12**, H_2O/MeOH 3:1; 57 mg; 0.95%)[16] and mussaenoside (**13**, H_2O/MeOH 3:1; 13 mg; 0.22%),[17] Figure 1.

The MeOH extract from *Cresentia alata* (23.9 g) was dissolved in MeOH and fractionated by CC (250 mL fractions) over silica gel 230–400 mesh using CH_2Cl_2/MeOH gradient as a mobile phase. Based on TLC, fractions were pooled in five groups. G-4, containing iridoids,[18] was further separated into pure compounds using CC eluting with CH_2Cl_2/MeOH yielding 6-*epi*-*O*-*p*-hydroxybenzoyl aucubin (**4**, CH_2Cl_2/MeOH 99:1; 175 mg, 0.73%)[19] and 6-*O*-(3″,4″-dimethoxycinnamoyl) catalpol (**6**, CH_2Cl_2/MeOH 98:2; 63 mg, 0.26%),[20] Figure 1.



	R	C7-C8	R^1	R^2
1	CH_2OH	$\Delta^{7,8}$	H	H
2	CH_2OH	$\Delta^{7,8}$	OH	H
3	PHB	$\Delta^{7,8}$	H	H
4	CH_2OH	$\Delta^{7,8}$	PMB	H
5	α-CH_2OH	–O–	OH	H
6	α-CH_2OH	–O–	3,4-DMB	H
7	β-Me,α-OAc	H	OH	OH

	R	C7-C8	R^1	R^2	R^3
8	CH_2OH	$\Delta^{7,8}$	H	H	COOH
9	$=CH_2$	7-β-OH	H	H	COOH
10	β-Me, α-H	7-β-OH	H	H	COOH
11	β-Me, α-H	7-β-OH	H	H	COOMe
12	β-H, α-Me	7-β-OH	H	H	COOMe
13	β-OH, α-Me	H	H	H	COOMe
14	β-H, α-Me	H	OH	OH	COOMe
15	β-H, α-Me	H	H	H	CHO
16	β-H, α-Me	H	H	OH	CHO

	$R^{1'}$	$R^{2'}$
PHB	H	OH
PMB	H	OCH_3
3,4-DMB	OCH_3	OCH_3

Figure 1. Chemical structures of iridoids **1**–**16**.

Iridoids, catalpol (**5**), 8-acetyl harpagoside (**7**), loganic acid (**10**), loganine (**11**) and penstemoside (**14**), were kindly donated by Prof. Soren Jensen.

To determinate the COX-1 and COX-2 inhibitory capacity of iridoids, in vitro assays were realized in 96 well plates using Cayman Chemical Company kits with catalogue number 765021. Absorbance of inhibitory reactions were measured in a Bio-Rad 680® microplate reader. Briefly, PGH_2 (prostaglandin H_2) is produced in this assay by COX's enzyme activity. It is then reduced with $SnCl_2$ to obtain $PGH_{2\alpha}$ which is measured via an enzyme immunoassay using a colorimetric antiserum that binds to all major prostaglandin products. Positive control of enzyme activity was assayed without an inhibitor, negative control was prepared by heat inactivation of enzyme. A curve of prostaglandins release was performed and indomethacin was tested as a reference compound. All iridoids and indomethacin were evaluated at a final concentration of 10, 35 and 100 μM and tested by triplicate (Table S1). The values were expressed as their mean (% of inhibition) ± SEM. Statistical differences between the treatments and control group were evaluated by ANOVA and Dunnett's *t*-tests; $p < 0.05$ was considered as significant.

Docking experiments were performed using Discovery Studio (DS) Client v2.5.0.9164 (2005–09) and Accelrys Software Inc. running on a HP xw4600 workstation (Processor x86 family 6 model 23 stepping 10 Genuine Intel 2999 ~MHz). The coordinates for the X-ray crystal structure for the enzyme ovine COX-1 and human COX-2 were obtained from the RCSB protein data bank (pdb). Subsequently, the ligand molecules were constructed using the Build Fragment tool. Both, the enzymes and ligand, were arranged using preparation tools in DS. The hydrogens were added to enzymes by keeping a pH of 7.5. Docking experiment on human COX-2 (pdb code = 1CX2) was carried out by CDOCKER in DS using Chemistry at HARvard Macromolecular Mechanics (CHARMm) forcefield. The ligand SC-558 was deleted and a sphere of 10 Å radius was generated to define the active site. CDOCKER generates random conformations of ligand within the active site. Therefore, docking was performed by simulated annealing with 2000 heating steps, 700 K heating target temperature, 500 cooling steps and 300 K cooling target temperature to generate 10 docked ligand poses. The enzyme–ligand bindings were evaluated by their CDocker interaction energies and CDocker energies in kcal/mol. Similar modeling experiments were carried out to investigate the binding with the COX-1 enzyme (pdb code = 1EQG) after deleting the ligand ibuprofen and defining a 10 Å radius active site.

Four major groups are accepted in the proposed classification for iridoids: (1) iridoid glycosides, which present a glycosidic link at C-1 or C-11; (2) simple iridoids with a methyl group at C-8, an extra carbon bonded to C-4 and can present a glycosidic link; (3) secoiridoids, with a cleaved C-7, C-8 bond, and finally (4) bisiridoids that are formed by the dimerization of iridoids and secoiridoids.[8] All iridoids evaluated in the present work are glycosylated in C-1 and belong to either the glycosylated iridoids (**1**–**7**)

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