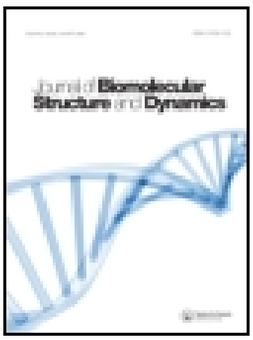

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Towards the understanding of the molecular basis for the inhibition of COX-1 and COX-2 by phenolic compounds present in Uruguayan propolis and grape pomace

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Running Head: Anti-inflammatory Activity of Phenolic Compounds

Abstract

Propolis and grape pomace have significant amounts of phenols which can take part in anti-inflammatory mechanisms. As the cyclooxygenases 1 and 2 (COX-1 and COX-2) are involved in said mechanisms, the possibility for a selective inhibition of COX-2 was analyzed *in vitro* and *in silico*.

Propolis and grape pomace from Uruguayan species were collected, extracted in hydroalcoholic mixture and analyzed. Based on phenols previously identified, and taking as reference the crystallographic structures of COX-1 and COX-2 in complex with the commercial drug Celecoxib, a molecular docking procedure was devised to adjust 123 phenolic molecular models at the enzyme binding sites.

The most important results of this work are that the extracts have an overall inhibition activity very similar in COX-1 and COX-2, i.e. they do not possess selective inhibition activity for COX-2. Nevertheless, 10 compounds of the phenolic database turned out to be more selective and 94 phenols resulted with similar selectivity than Celecoxib, an outcome that accounts for the overall experimental inhibition measures.

Binding site environment observations showed increased polarity in COX-2 as compared with COX-1, suggesting that polarity is the key for selectivity. Accordingly, the screening of molecular contacts pointed to the residues: Arg106, Gln178, Leu338, Ser339, Tyr341, Tyr371, Arg499, Ala502, Val509 and Ser516, which would explain, at the atomic level, the anti-inflammatory effect of the phenolic compounds. Among them, Gln178 and Arg499 appear to be essential for the selective inhibition of COX-2.

Keywords: propolis, grape pomace, phenols, selective anti-inflammatory activity, COX-1, COX-2, molecular docking

1 - Introduction

Propolis is a natural product with a high content of polyphenols (Martínez-Valverde, Periago, & Ros, 2000) and is produced by bees that collect a resinous substance from trees, which later on they take to the hive and process adding other elements (Farré, Frasquet, & Sánchez, 2004). Properties reported for propolis include: antioxidant, antimicrobial, antiviral, fungicide, wound healing, anaesthetic and anti-inflammatory (Peña, 2008). Propolis' chemical

composition is quite complex and depends on the plant source from where it is generated. It is composed of 45 - 55 % resins, 7 - 35 % bee glue, 5 - 10 % essential and volatile oils, 5 % pollen and 5 % diverse compounds (organic and mineral) (Farré et al., 2004).

In propolis, more than 160 compounds have been identified, of which 50 % are polyphenols (Farré, 2004), the most abundant being: flavonoids (flavones, isoflavones, flavonones), phenolic acids (caffeic acid, cinnamic acid and others), aromatic aldehydes (vainillin and isovainillin), coumarines and phenolic triglycerides.

On the other hand, grape pomace is a by-product of winemaking industry generally destined to the extraction of the remaining alcohol by distillation. It is composed of grape skins and seeds (Flanzy, 2002). In grape pomace, pigments, phenolic acids, flavonoids and tannins are found, such as epicatechin, catechin, quercetin, myricetin, kaempferol and resveratrol (Ferreira, Sellés, & Valenzuela, 2002; Flanzy, 2002). In particular, these phenolic acids, flavonoids and resveratrol, have shown multiple biological properties such as antioxidant capacity and anti-thrombosis (Rockenbach et al. 2011; Pace-Asciak, Hahn, Diamandis, Soleas & Goldberg, 1995).

A mixture of both, propolis and grape pomace would have a high content of phenolic compounds, with a very wide structural spectrum (Kumazawa, Hamasaka, & Nakayama, 2004; Kumazawa, Kajiya, Ishii, Hamasaka, & Nakayama, 2002; Paulino-Zunini et al., 2010; Silva et al., 2011; Boido, Alcalde-León, Carrau, Dellacassa, & Rivas-Gonzalo, 2006). This trait would confer the mixture with antioxidant and anti-inflammatory activities, among others, stabilizing free radicals and inhibiting the activity of enzymes related to oxidative stress and inflammation, therefore controlling those processes.

Same evidences indicate that phenols could have anti-inflammatory capacity. Inflammation is a multi-factorial process. It reflects the response of the organism to the various stimuli and is related to many disorders such as arthritis, asthma, and psoriasis which require prolonged or repeated treatment. Cyclooxygenase (COX), the rate limiting enzyme of the eicosanoids biosynthetic pathway, catalyzes the conversion of arachidonic acid to important anti-inflammatory mediators such as prostaglandins (PGs), prostacyclin (PGI) and thromboxane (TXA2) (Vane 1996). It is well known that cyclooxygenase exists in two isoforms, cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) (Frank & Fries, 1991). COX-1 is a constitutive enzyme and is responsible for the production of cytoprotective prostaglandins in the gastrointestinal tract and pro-aggregatory thromboxanes in blood

platelets. However, COX-2 is an inducible enzyme, which is produced in response to the release of several pro-inflammatory mediators such as TNFα, IL-6, IL-1, LPS, carragenan, 12-O-tetradecanoylphorbol-13-acetate (TPA), and histamine.

Since COX-2 is involved in the inflammation process and the resulting pain, the inhibition of its enzymatic activity would be of therapeutic value. It is, in fact, the target enzyme for the anti-inflammatory activity of non-steroidal anti-inflammatory drugs (NSAIDs) (Meade, Smith, & DeWitt, 1993). These molecules include aspirin and indomethacin which are non-selective anti-inflammatory agents and inhibit both COX-1 and COX-2. Aspirin inhibits COX-1 more strongly than COX-2 and inhibition of COX-1 by aspirin reduces the production of PGE2 and PGI2, which has an adverse ulcerogenic effect (Mitchell, Akarasereenont, Thiemeromann, Flower, & Vane, 1993). The COX-2 gene expression is induced in inflammation and other pathologies, such as cancer proliferation and has led to the development of COX-2 selective inhibitors to improve the therapeutic potency and reduce the classical side effects associated with the use of conventional NSAIDs (Dannhardt & Kiefer, 2001). In fact, selective COX-2 inhibitors (coxibs) with better safety profile have been marketed as a new generation of NSAIDs (Tally et al., 2000). One of best known NSAIDs anti-inflammatory drug is Celecoxib, used to treat pain or inflammation caused by many conditions such as arthritis, ankylosing spondylitis, and menstrual pain. Celecoxib, and other coxibs, may cause life-threatening heart or circulation problems such as heart attack or stroke, especially in long term uses (Dogne, Supuran, & Pratico 2005). Therefore, development of novel compounds having anti-inflammatory activity with an improved safety profile is still of paramount importance.

The objectives of the present work are as follows: a) to analyze the COX-1 and COX-2 inhibitory capacity of propolis and grape pomace extracts, and their mixtures; b) to build a database containing the tridimensional structure of the previously identified phenols in the extracts (Kumazawa et al., 2004; Paulino-Zunini et al., 2010; Silva et al., 2011; Boido et al., 2006); c) to gain a better understanding on how and why phenols may interact with COXs enzymes; d) to examine the protein-ligand interactions of the phenolic compounds docked at COX-1 and COX-2 active sites and relate these observations with the inhibitory activity in order to identify the molecular characteristics that would improve the specific inhibition of COX-2.

2 - Materials and Methods

2.1 In vitro studies

2.1.1 Chemicals and Instruments

For the COX inhibition test, a *Cayman* kit (Item Chemical's ACE™EIA Kits No.560131) was used (Cayman, 2012).

2.1.2 Samples

Propolis samples from different geographical Uruguayan origins and grape pomace samples of Tannat, Carbernet-Sauvignon, Merlot and Arinarnoa were collected from 2010 to 2013.

2.1.3 Extraction of phenols

2.1.3.1 Propolis extraction

Samples of 1g weight were separately extracted. Waxes were removed by Soxhlet in 200 mL hexane during one hour and 12 refluxes. After evaporating the hexane and drying, they were grinded and phenols were extracted during one hour at 75°C. Finally, 25 mL of extract were filtered and diluted to 50 mL. The liquid extracts were stocked at 4°C-until their analysis.

.2.1.3.2. Grape pomace extraction

Frozen grape pomace samples were submitted during 24 hours to drying procedure at a temperature of 60 °C. The dry grape pomace samples were grinded and stocked in darkness. Phenols were extracted by reflux in a mixture of ethanol-water (80-20 v/v) during 2 hours at 50 °C. After filtering, the extracts were conserved at 4 °C in darkness until their use.

2.1.4 In vitro anti-inflammatory activity by COX-1 and COX-2 inhibition.

The COX Inhibitor Screening Assay directly measures PGF2α produced by $SnCl_2$ reduction of COX-derived PGH_2 in the presence of 20µg/mL of each extract or solvent as control. The prostanoid product is quantified via enzyme immunoassay (EIA) using a broadly specific antibody that binds to all the major prostaglandin compounds, according to the

manufacturer (Xie, Chipman, Roberston, Erikson, & Simmons, 1991; Blobaum & Marnett, 2007).

2.1.5 Total phenolic content quantification

Total phenolic content was determined using the method described by Singleton, Orthofer, and Lamuela-Raventos (1999).

2.2 In silico studies

All calculations and procedures were carried out by means of the Molecular Operating Environment MOE 2011.10 (Chemical Computing Group, 2010).

2.2.1 The ligands to be docked: phenolic database building

All phenolic structures identified in propolis (Paulino-Zunini et al., 2010, Silva et al., 2011) and grape pomace (Boido et al., 2006) were modeled and used to evaluate the $logP_{o/w}$ and the Lipinski indexes. Their structure refined by energy minimization, employing the MMF94x force field (Halgren, 1999).

2.2.2 Selection of a receptor and site

Many crystallographically resolved 3D structures are available for COX-1 and COX-2, with resolutions in the range of 2-3 Å. Two criteria were applied to select the enzymatic receptor structures: 1) the existence of a co-crystallized ligand similar in function to the endogenous ligand and 2) the best possible x-ray crystallographic resolution. As a result, the crystallographic coordinates of the homodimer COX-2 with a X-ray diffraction resolution of 2.4 Å (Protein Data Bank ID: 3LN1) (Wang et al., 2010) and those of the homodimer COX-1 at 2.75 Å resolution (Protein Data Bank ID: 3KK6) (Rimon et al., 2010) were selected as the receptor structures. Both crystallographic enzyme structures are co-crystallized with Celecoxib. Heavy atom charges and hydrogen atoms were fixed using an MMFF94x force field (Halgren, 1999).

To select a ´site´ for docking in both COX-1 and COX-2, the Site Finder utility in MOE suite was employed.

2.2.3 Docking and filtering with a site pharmacophore

The final step of the *in silico* study was a flexible docking procedure of the phenol database at COX-1 and COX-2 active sites.

First, a validation of the docking protocol was performed, using the crystallographic position of Celecoxib in COX-1 (3KK6 crystal) and COX-2 (3LN1 crystal), as references. The Celecoxib based pharmacophore, together with Alpha PMI, Alpha Triangle, Proxy Triangle and Triangle Matcher algorithms were tested as placement methods. The scores of the docked conformations were calculated with the Affinity ΔG function which measures the enthalpic contribution to the free energy of binding (Chemical Computing Group, 2010).

The docking procedure was applied to the database previously built containing all phenols identified in propolis and grape pomace, using the best placement methods found in the validation procedure and the same scoring function. Thirty best poses were retained for each ligand compound.

A receptor pharmacophore was built: first, a molecular surface of the receptor was created around the Celecoxib molecule (crystallographic structure), both for COX-1 and COX-2. The molecular surface was constructed according to (Connolly, 1996) with a water radius of 1.4 Å. As a second step, the receptor pharmacophore was created, for both enzyme binding sites, using the Polar-Planarity-Charge-Hydrophobicity (PPCH) scheme. Of all possible features to be included, we selected only those related with the polarity of the surface. In this situation, four 2 planar hydrophobic features (HydP), one non planar hydrophobic (HydS) and one non planar hydrogen acceptor (accS) were retained. HydS was in the CF3 atomic group, HydP were located in the imidazolic ring and in one of the benzene rings, and the accS was mapped to the sulphonamide group of Celecoxib. After selection, the feature sizes were adjusted to fill the pocket.

Finally, after docking, a filter with the site pharmacophores was applied (one for each enzyme) and as a result, a set of phenols whose placement displayed a coincidence with that of crystallographic Celecoxib in COX-1 and in COX-2, was obtained.

2.2.4 Ligand interactions

The Ligand Interactions tool in MOE suite was utilized to visualize the most significant molecular interactions (including solvent accessible surface areas) between the docked ligands and the binding site residues, both for COX-1 and COX-2. Two cut-off distances of 4.5 Å and 5.8 Å were established. Contacts within the short cut-off radius were

considered as ´close´ contacts and those between both cut-off radii were considered as ´weak´ contacts.

3 - Results and discussion

3.1 *In vitro* studies

The results of COX-1 and COX-2 enzyme inhibition by the phenolic samples as well as the total phenol contents are summarized in Table 1. Samples numbered 159, 161 and 306 to 315 are from propolis, samples 304, 305 and 333 are from grape pomace and the samples 316 to 319 are mixtures.

Samples 307 and 309 proved to have the best inhibition capacity for COX-1 while samples 308, 304 and their mixture were the best for COX-2. In general, the samples of propolis and grape pomace showed a lack of specificity towards the inhibition of COX-2 since they are able to inhibit both COX isoforms to a similar extent. Inhibition ranges (%) were 39.4 – 72.7 for COX-1 and 24.7 – 77.1 for COX-2. Inhibition averages for COX-1 and COX-2 were very much alike considering all samples (61.2 ± 1.7 and 58.8 ± 3.6, respectively). No significant variations between the overall inhibition of propolis samples and grape pomace samples were obtained either.

One-way ANOVA with Dunnett's test was performed using GraphPad Prism version 5.01 Software (GraphPad Software Inc, 2007). A correlation would be significant if its p-value is less than 0.05 according to Pearson (D'Agostino, 1986).

The total phenolic content (%GAE) in grape pomace (samples 304, 305 and 333) ranged from 3.9 to 7.6 % GAE, and in propolis extracts (samples 159, 161 and 306-315) ranged from 4.0 to 15.0 % GAE, demonstrating that propolis is more concentrated in phenols. No correlation was found between the polyphenol contents obtained by Folin-Ciocalteu and percentages of COX-1 and COX-2 inhibition ($p = 0.7950$ and 0.3920, respectively).

The drug-likeness Lipinski index (see Supplementary Material A) resulted with positive values for all phenols in propolis while only 22% of phenols present in grape pomace extracts have positive values. This index describes in a very simple way whether a given compound is likely to be an active drug in humans when administered orally. In particular, a positive value indicates a good compound bioavailability.

This implies that not only the total content but the kind of phenols contained in the extracts are critical for enzyme inhibition and in consequence to promote anti-inflammatory effects in humans.

3.2 *In silico* studies

3.2.1 Site finder

Sixty eight putative binding sites were detected for the COX-2 dimer. Two of them are located at the position of co-crystallized Celecoxib hence indicating that they correspond to the active sites of the enzyme (one for each monomer). A similar scenario was found for COX-1: sixty one putative binding sites were detected and two of them corresponded to the active sites of the enzyme. For both COX-1 and COX-2, the best ranked sites were the active sites. This result, together with the experimental assays made with pure phenols in COX-1 and COX-2 (Badieyan, Moallem, Mehri, Shahsavand & Hadizadeh, 2012; Ya-Di Li et al 2011) and the fact that Celecoxib crystallizes in the active sites of both enzymes, as shown by the corresponding crystallographic complexes, suggest that phenols are competitive inhibitors of COX-1 and COX-2.

3.2.2 Sequence and tridimensional structure comparison

Selective COX-2 inhibitors exhibit time-dependent inhibition of COX-2 but not COX-1. At the entrance of the COX channel, Arg120, Glu524, Tyr355 and His90 form a network of hydrogen bonds that act as a gate to the binding site (Zarghi & Arfaei, 2011). NSAIDs generally bind at the upper portion of the COX channel located near Tyr385 and Arg120 which is present at the mouth of the COX channel. The carboxyl moiety of some acidic NSAIDs such as flurbiprofen interacts with Arg120 in both COX isoforms, via hydrogen bonding or electrostatic interactions (Marnett, Rowlinson, Goodwin, Kalgutkar, & Lanzo, 1999). The remaining ligand-protein interaction is hydrophobic. Structural differences within the binding sites of the COX isoforms have been exploited to design selective COX-2 inhibitors. To have a deeper understanding of the determinants of specificity towards COX-2, both, COX-1 and COX-2, were studied by comparison of their sequences and further tridimensional analysis of their binding behavior towards those phenols that display better COX-2/COX-1 scoring energy ratio (see below).

The Protein Data Bank COX-1 sequence has 587 aminoacids length and that of COX-2, 553. When aligned, the residues making contact with Celecoxib, in both enzymes, were surveyed. 26 aminoacids were found in a 4.5 Å sphere around the Celecoxib molecule. The result is shown in Table 2.

The result of the sequence analysis is shown in Figure 2. Both sequences share a global similarity of 65%. When only the 26 aminoacids making contact with Celecoxib are considered, only two of them are non-conserved, raising the local sequence similarity to 92%.

Even though most residues contacting Celecoxib are identical in the two enzymes, there are subtle differences that may help understand the different behavior of both binding sites.

Ile492 in COX-2 is replaced in COX-1 by a valine residue and, in agreement with the observation of Zarghi and Arfaei (2011). Ile has a $\log P_{o/w}$ of 0.4340 while Val has a value of 0.8760 indicating a more hydrophobic environment in COX-1. The non conservative change Ser/Ala485 is further evidence of an increase in polarity for the COX-2 binding site. In the same line, two polar residues are in the COX-2 contact sphere (Thr63 and Tyr317) but not in the COX-1 one. Finally, Asn282 in COX-2 makes contact with Celecoxib while the corresponding aspartic residue in COX-1 is not within the contacting surface in COX-2. All these substitutions would act by increasing the polarity of the celecoxib environment in COX-2 as compared with COX-1.

3.2.3 Docking validation with Celecoxib

In Table 3, the scores and RMSD values for the validation docking of Celecoxib in COX-2 are listed. In view of these results, the best placement method (i.e., the one that yielded the orientations of Celecoxib which matched best those of the crystallographic structure) was Alpha Triangle. In the case of COX-1, the best match with crystallographic Celecoxib was achieved with Triangle Matcher (results not shown). Triangle Matcher is a variant of Alpha Triangle, thus all subsequent dockings (i.e. applied to the phenol database) were conducted using Alpha Triangle for COX-2 and Triangle Matcher for COX-1 considering them as similar algorithms.

3.2.4 Docking and site pharmacophore filtering of the phenolic database

The results for the best scored phenol compounds in COX-1 and COX-2 as well as their $\log P_{o/w}$ and Lipinski indexes are presented in Supplementary Material A and

summarized in Table 3. The structures of the main molecular scaffolds are shown in Supplementary Material B.

3.2.4.1 Comparison of theoretical and experimental binding energies

Recently, the Celecoxib micro molar IC_{50} in COX-1 (13.02 μM) and in COX-2 (0.49 μM) were published (Badieyan, Moallem, Mehri, Shahsavand, & Hadizadeh, 2012). Similar data are available for myricetin, luteolin and quercetin (Badieyan, Moallem, Mehri, Shahsavand, & Hadizadeh, 2012, Ya-Di et al., 2011).

To be able to compare the experimental and *in silico* measurements of binding energy, a competitive inhibitory mechanism is assumed (as mentioned in section 3.2.1) and in consequence, the approximation of Cheng-Prusoff (Cheng Y, Prusoff WH, 1973) was applied. For enzymatic reactions, the Cheng-Prusoff equation is:

$$K_i = IC_{50}/(1+([S]/K_m) \quad [1]$$

where K_i represents the binding affinity of the inhibitor, IC_{50} is the concentration of competing ligand which displaces 50% of the specific binding of the ligand in the experimental assay, [S] is a fixed substrate concentration and K_m is the concentration of substrate at which enzyme activity is at half maximal. As the same experimental conditions are accepted for all inhibitors, the experimental binding free energies may be calculated from IC_{50} using the following equation:

$$\Delta G_{bind} = RT\ln IC_{50} \quad [2]$$

where *R* is the ideal gas constant 8,31 x 10-3 KJ/mol and *T* is the temperature in *K* degrees (298 K is used), (Zhong H et al, 2013).

If the experimental and in silico values for the binding energies of myricetin, luteolin, quercetin and Celecoxib are plotted against each other using data presented in Table 4, a correlation of R^2=0.9929 is found which demonstrates the striking concordance between the experimental and in silico methods. This result validates our methodology approach and permits us to consider the docking scores as reasonable approximations to the experimental

binding energies in situations where the latter are not available as it is the case for most phenol compounds assayed. In this context, the relationship between experimental and theoretical scores can be described by equation [3] and used to predict the experimental binding free energies by means of the scores calculated by docking:

$$\Delta G_{score} = 0.52\,\Delta G_{exp} - 20.24 \quad [3]$$

The experimental results indicate that the binding of Celecoxib is roughly 8 kcal/mol more favorable in COX-2. This energy gap may be taken as a benchmark for a given compound (in particular belonging to the phenol sample studied here) to be considered a good inhibitor (at least as selective as Celecoxib) of COX-2.

As already mentioned in the introductory section, there has been enough evidence pointing to serious gastrointestinal complications and cardiovascular thrombotic events caused by the administration of Celecoxib in osteo and rheumatoid arthritis patients. These side effects should be caused by the inhibition of COX-1 by Celecoxib (Fabule & Adebajo, 2014). It remains to be observed in further research if phenols are able to avoid those side adverse effects.

3.2.4.2 Phenolic database annotation by Selectivity Index

Taking into account all docked phenols (Supplementary Material A), the COX-2/COX-1 scores ratio was calculated as a selectivity index (SI) and plotted in Figure 3.

SI scores of the whole database were divided into three categories: high (from 1.94 to 1.43), medium (from 1.37 to 0.84) and low (0.81 to 0.35). Only 10 of 123 analyzed phenols display a high SI index. However, 94 of them are in the medium level, in the range of Celecoxib (SI = 0,94) showing that many of the compounds would possess an inhibition selectivity towards COX-2 similar to Celecoxib. When the SI is plotted against the $\log P_{o/w}$ (Figure 3c), a correlation is evidenced: as hydrophilicity increases, the SI becomes higher. This could be related now with our previous observation of polarity differences in the binding sites (Figure 1 and 2 and Table 2).

A plot of SI colored ranges is shown in Figure 3a. In Figure 3b, a similar plot separating the phenols by their natural origin makes apparent that the phenols with higher SI values are found in grape pomace. From these results, one can conclude that compounds belonging to the grape pomace have higher selectivity than those present in propolis. As

mentioned above, those with greater selectivity would have a tendency to be more hydrophilic considering the Log $P_{o/w}$ index.

The 10 best ranked SI scores (Table 5) correspond to: two phenolic acids (Z-fertaric and E-fertaric acids), four anthocyanines (Delphinidin-3,7-diglucoside, Malvidin-3-O-(6-acetyl)-glucoside, Malvidin-3,7-diglucoside and Petunidin-3-O-(6-acetyl)-glucoside), three derivatized flavones (Syringetin-3-O-glucoside, Laricitrin-3-O-glucoside and Myricetin-3-O-galactoside) and one flavone (Pinobanskin).

The Z and E fertaric acids (condensation of ferulic and tartaric acid) are present in high concentrations in the grape skin and in low concentrations in the seeds (Kashif, Maltese, Hae Choi & Verpoorte., 2009). The Z conformation appears to be the most selective. On the contrary, the four anthocyanins are present in the grape seed in high concentrations and are less concentrated in the skin (Kashif et al., 2009). Other compounds such as the 3-O-Glycosides flavonols: Syringetin 3-O-glucoside and laricitrin 3-O-glucoside present higher SI but they are present in low concentrations in the grape pomace (Kashif et al., 2009).

Two compounds present in propolis, namely pinobanskin and myricetin-3-O-galactoside possess a high SI index. Both of them are relatively abundant flavonoids in Uruguayan propolis (Silva et al. 2011) and from other geographical sources (Bankova, 2005; Falcão et al., 2010). In addition, quercetin, myricetin and luteolin, which are at least as good inhibitors of COX-2 as Celecoxib (check SI indexes in Table 5), have been detected in propolis samples in various quantities. To relate this observation with the experimental available data, it must be mentioned that the same Enzyme Immuno Assay (EIA) applied to the extracts was used to assess the binding free energy of celecoxib, myricetin, quercetin and luteolin (Badieyan, Moallem, Mehri, Shahsavand & Hadizadeh, 2012; Ya-Di Li et al 2011) (see Table 4). Even if the phenolic composition of the extracts is very complex, a similar behavior tendency as compared with that of pure phenols may be envisaged in terms of competitive inhibition. Hopefully, future assays will reveal that grape pomace and/or propolis samples contain high concentrations of the 10 best ranked phenols in terms of SI index (see above). The higher polarity and net charge found in these phenols could anticipate - in view of our conclusions - the discovering of better and more selective COX-2 inhibitors.

3.2.4.3 Interaction analysis at the binding sites

The residues making closer contacts with Celecoxib and with those phenols with the highest SI were analyzed. Since this set of amino acids is considered relevant for the functionality of both enzymes, in a second step of the analysis, the distances between

hydrogen donor and acceptor were measured for the phenols with the ten best SI values for both COX-1 and COX-2.

A set of amino acids in COX-2 establish conspicuous contacts with Celecoxib, namely Arg106, Gln178, Leu338, Ser339, Tyr341, Tyr371, Arg499, Ala502, Val509 and Ser516. For the COX-1 interaction region we observed the same kind of residues except for Arg499 in COX-2, replaced by His513 in COX-1, Ala502 that is replaced by Ser516 and another conservative replacement of Val509 by Ile523.

In Figure 1, most of the residues which showed interactions with the best docked compounds in COX-2 and COX-1 are depicted. All of them are located near the peroxidase site previously described (Zarghi & Arfaei, 2011). The relevance of Tyr371 has been reported elsewhere (Rowlinson S W. 2003).

In Table 6, the ten best ranked phenols, in terms of SI index, were arranged from the right to the left following SI. Then, all contacts were analyzed and classified depending on the nature (H-bond or another kind of non bonded interaction) and the distance.

If our only concern were to have an anti-inflammatory molecule regardless of selectivity, it would suffice for a ´good´ inhibitor candidate to have strong interactions with COX-2, i.e. contacts at less than 4.5 Å. These are the yellow cells in Table 6. Now, if we are interested in selectivity as well, in addition to the closer contacts in COX-2, weak (or non-existent) contacts in COX-1 should be taken into account. These are the green cells in Table 6.

Arg499 appears to be crucial for binding at COX-2 since, in all cases but one (pinobanskin), it establishes an H-bond with the ligands. This residue is replaced by a histidine (His513) in COX-1 and, as it is apparent in Figure 5 and Table 6, His513 is not able to establish strong contacts with phenols in COX-1. The role of Arg499 is consistent with the above mentioned differences in the binding sites of COX-1 and COX-2: the increased polarity in the latter is fundamental for selectivity.

Arg106 also contacts most of the ligands in the phenol sample. This observation is in agreement with Marnett (1999) and Zarghi (2011). However, while the interaction with Arg499 is specific for COX-2, this arginine residue (Arg120 in COX-1) also establishes close contacts in COX-1.

In the case of Z-fertaric (the phenol with the best selectivty index), Gln178, Arg499, Leu338, Ser339 and Ser516 contribute to a selective interaction with COX-2. This is in agreement with a large difference in the scoring energies between COX-2 (-36 kJ/mol) and COX-1 (-18 kJ/mol). Additionally, a strong contact in COX-2 is detected with Tyr341, a

conserved residue (Tyr355 in COX-1). Clearly, this interaction is not enough to counterbalance the binding selectivity conferred by the other contacts.

The E-fertaric acid has some differences with respect to its Z isomer establishing specific contacts with Gln178, Leu338, Ser339, Tyr341 and Tyr371. It also gains strong and non specific H bonds with Arg106/120.

Syringetin-3-O-glucoside, with a selectivity index of 1.84 displays specific contacts with Gln178, Leu338, Tyr341, Tyr371 and Arg499 in COX-2. A strong and non specific contact is detected with Arg106/120.

Delphinidin-3,7-diglucoside makes specific contacts with Gln179, Ser339, Tyr341 and Arg499. A non-specific and strong contact in both enzymes is detected with Ser516. It is interesting the fact that some of those contacts are complemented with hydrophobic/aromatic interactions. Non specific contacts with Arg106/120, Tyr341/255 and Ser516/530 are also detected.

In Figures 4 and 5, the interactions of five of the analyzed compounds (four molecules belonging to the grape pomace extracts and the flavone pinobanksin from propolis) are shown in COX-2 and COX-1 respectively.

Figure 4 is a three-dimensional picture of the binding mode of Z and E-fertaric acids, delphinidin-3,7-diglucoside, syringetin-3-o-glucoside, myricetin-3-O-galactoside and pinobanksin with the aim of helping the visualization of the selectivity basis for COX-2. In Figure 5, the same molecules are observed in the binding site of COX-1. All observations made from Table 6 could be confirmed in these tridimensional views of the sites.

Pinobanksin, the smallest of the 10 molecules with the highest SI indexes, has close and strong interactions with Leu338, Tyr371, Val509 and Ser516 in COX-2. Similar contacts with corresponding residues in COX-2 are not observed. In addition to the hydrogen bonds, pinobanksin establishes a stacking π-cation interaction with Arg120 in COX-1. Arg120 is important for stabilizing binding in COX-1 but not in COX-2. It is noticeable that pinobanksin is the only phenol of those analyzed in Table 6 with no contacts with Arg499.

Finally, in the right side of Figure 4 and 5, the colorful molecular surfaces surrounding the phenols clearly suggest the role of polarity for binding. This is more apparent in Figure 4 (binding to COX-2): in the region near the basic Arg106 and Arg499, there is always a corresponding blue colored surface indicating that a positive distribution of charge in the enzyme is necessary for a strong binding.

Hitherto, the analysis of contact was made taking into account mainly the residue side chain atoms. To have a complete picture of all molecular interactions (considering the backbone atoms as well as the side chains) at 4.5 Å distance, the sequence alignment (in the region of the binding sites) previously built (see Figure 2) was used to annotate in bold characters all the contacts (H bond and others) with the phenol compounds. This analysis is presented in the Supplementary Material C. The stretches MKYVLTS**R$_{106}$**; VIEDYVQH**L$_{338}$S$_{339}$**G**Y$_{341}$**HFKL; **F$_{367}$**NTL**Y$_{371}$**H**W$_{373}$**; P**R$_{499}$**PD**A$_{502}$**IFGETM**V$_{509}$**ELGAPF**S$_{516}$**LKGLM and $_{71}$PNTVHYILT emerge as important regions for the selective inhibition. It is worth noting that all the already identified residues namely Arg106, Leu338, Ser339, Tyr341, Tyr371, Arg499, Ala502, Val509 and Ser516 (marked in bold characters in the sequences, see the supplementary material C) are included in these regions. The exception is Gln178 which appears isolated and not within any of the observed contacting regions. The other observation is that the **Phe$_{367}$** and the **Trp$_{373}$** (included in the **F$_{367}$**NTL**Y$_{371}$**HW stretch) were not detected in the previous molecular interaction analysis (Figure 1 and Table 6). However, as can be seen in the supplementary material C these two residues make contacts with all phenols but Z-fertaric. Therefore, some main chain atoms of these residues may act as specific contacts with COX-2.

In summary, there is a set of residues that a potential anti-inflammatory molecule should make contacts with, comprising: Arg106, Gln178, Leu338, Ser339, Tyr341 Tyr371, Arg499, Ala502, Val509 and Ser516. Yet, if one is looking for selective inhibition of COX-2, it seems that Arg499 and Gln478 are the key residues. Additionally, the F_{367} and the Trp_{373} could eventually contribute to the specificity.

4. Conclusions

The propolis and grape wine extracts proved to have in vitro anti-inflammatory effect, a feat that was verified *in silico* in terms of binding energies. Indeed, the *in vitro* assays with 20µg/mL of each extract, showed percentages in the range 39,4 - 72,7% of COX-1 inhibition and 24,7 - 76,8% of COX-2 inhibition.

The validated docking procedure predicted energy scores for the studied compounds, some of them showing a good correlation with experimental binding free energies calculated from IC_{50} values in COX-2. Moreover, the docking results in both COX-1 and COX-2 were consistent with the quite similar inhibitory behaviors found for the extracts in the *in vitro* assays.

These fairly equivalent inhibitory behaviors show that, overall, the assayed samples do not possess selective inhibition activity for COX-2. Nevertheless, some compounds of the phenolic database turned out to be more selective than Celecoxib. In particular, ten phenol derivatives showed higher SI indexes: two acids (Z-fertaric and E-fertaric acids), four anthocyanines (delphinidin-3,7-diglucoside, malvidin-3-O-(6-acetyl)-glucoside, malvidin-3,7-diglucoside and petunidin-3-O-(6-acetyl)-glucoside), three derivatized flavones (syringetin-3-O-glucoside, laricitrin-3-O-glucoside and myricetin-3-O-galactoside) and one flavone (pinobanskin). Moreover, ninety four phenols resulted with similar SI than Celecoxib, a result that accounts for the overall experimental inhibition measures.

Compounds belonging to the grape pomace have higher selectivity than those present in propolis. In contrast, as can be seen in Table 1, the total phenolic content is higher in propolis than in grape pomace. Then, it could be conjectured that the similar overall level of inhibition for COX-2 of propolis and grape pomace extracts is likely the consequence of two different factors that counterbalance each other: phenols contained in grape pomace extracts are less concentrated but more powerful inhibitors than those found in propolis.

Binding site environment observations showed increased polarity in COX-2 as compared with COX-1, suggesting that polarity could be the key for selectivity. In agreement with this observation, the screening of molecular contacts that could favor the binding at COX-2 pointed to a set of residues namely: Arg106, Gln178, Leu338, Ser339, Tyr341, Tyr371, Arg499, Ala502, Val509 and Ser516, which would explain, at the atomic level, the anti-inflammatory effect. Among them, Gln178 and Arg499 appear to be essential for the selective inhibition of COX-2.

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CAPTIONS FOR THE FIGURES

Figure 1. Right side: ribbons rendering of the aligned chain A three-dimensional structures of COX-1 (green) and COX-2 (red). Structures correspond to the crystallographic coordinates. Celecoxib docked structures are rendered in stick models, pink (in COX-1) and yellow (in COX-2). Left side: a zoom view of the active site showing residues contacting Celecoxib.

Figure 2. COX-1 (upper line) and COX-2 (bottom line) sequences aligned. Binding site residues contacting with Celecoxib are colored depending on their polarity: blue (positive charged), orange (polar) and green (hydrophobic). Asterisk marks are shown every ten positions.

Figure 3. **3a.** Selectivity Index (COX-2/COX-1 scoring ratio) for the 123 phenols detected in propolis and grape pomace. Colore codes: purple (high SI range); blue (medium SI range) and brown (low SI range). **3b.** SI distribution as a function of the natural origin. **3c.** SI as a function of hydrophobicity (log $P_{o/w}$).

Figure 4. Ligand Interactions (left) and Surface Area graphs (right) for the best SI ranked compound docked in COX-2 binding site. A) (Z)-Fertaric acid and (E)-Fertaric acid in yellow and orange respectively; B) Dephinidin-3,7-diglucoside in red; C) Syringetin-3-O-glucoside in green; D) Myricetin-3-O-galactoside in pink. E) Pinobanksin in black. Colors of the surface areas evidence electrostatic nature: blue (positive), red (negative) and white (neutral).

Figure 5. Ligand Interactions (left) and Surface Area graphs (right) for the best SI ranked compound docked in COX-1 binding site.: A) (Z)-Fertaric acid and (E)-Fertaric acid in yellow and orange respectively; B) Dephinidin-3,7-diglucoside in red; C) Syringetin-3-O-glucoside in green; D) Myricetin-3-O-galactoside in pink. E) Pinobanksin in black. Colors of the surface areas evidence electrostatic nature: blue (positive), red (negative) and white (neutral).

Fig. 1

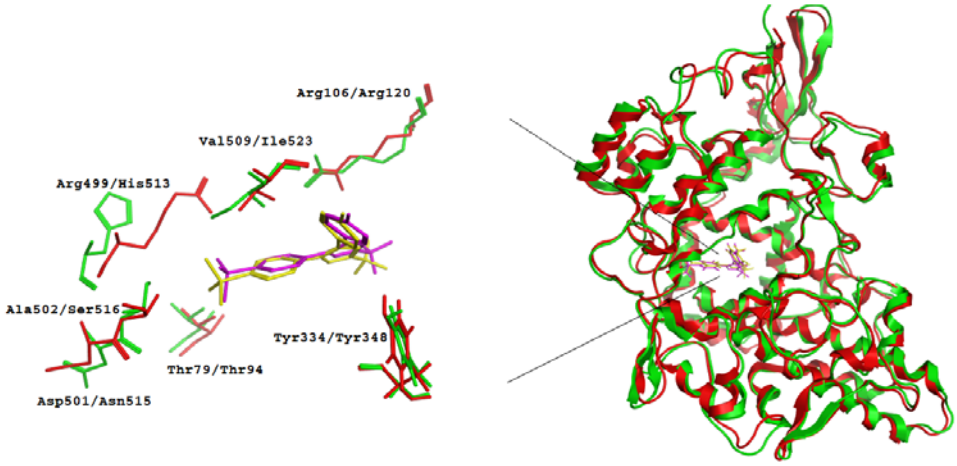


Fig. 2

Fig. 3

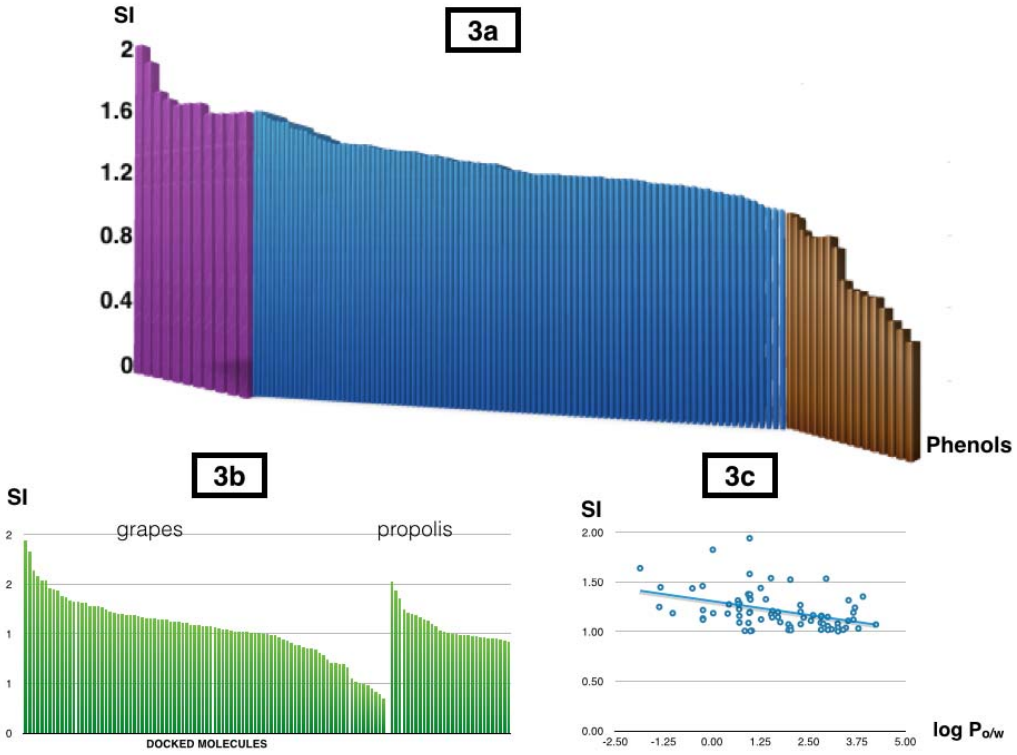


Fig. 4

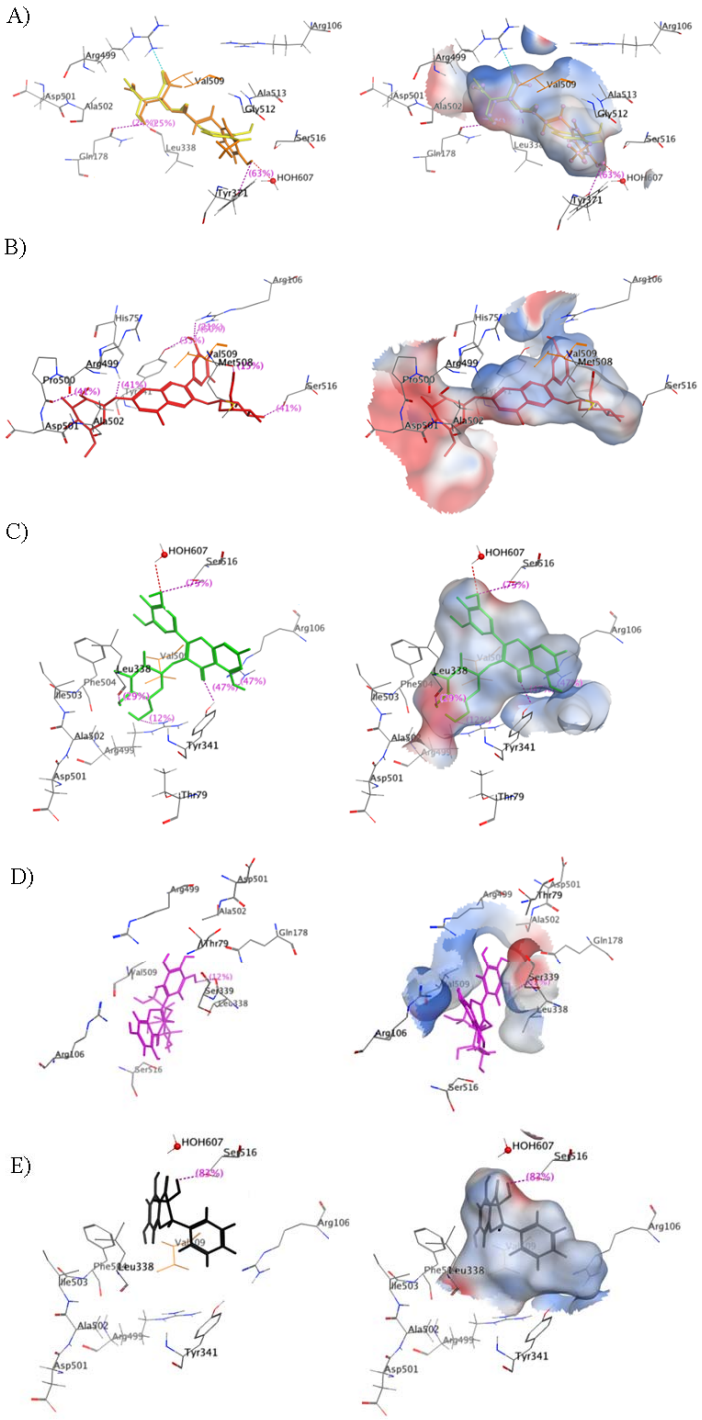


Fig. 5

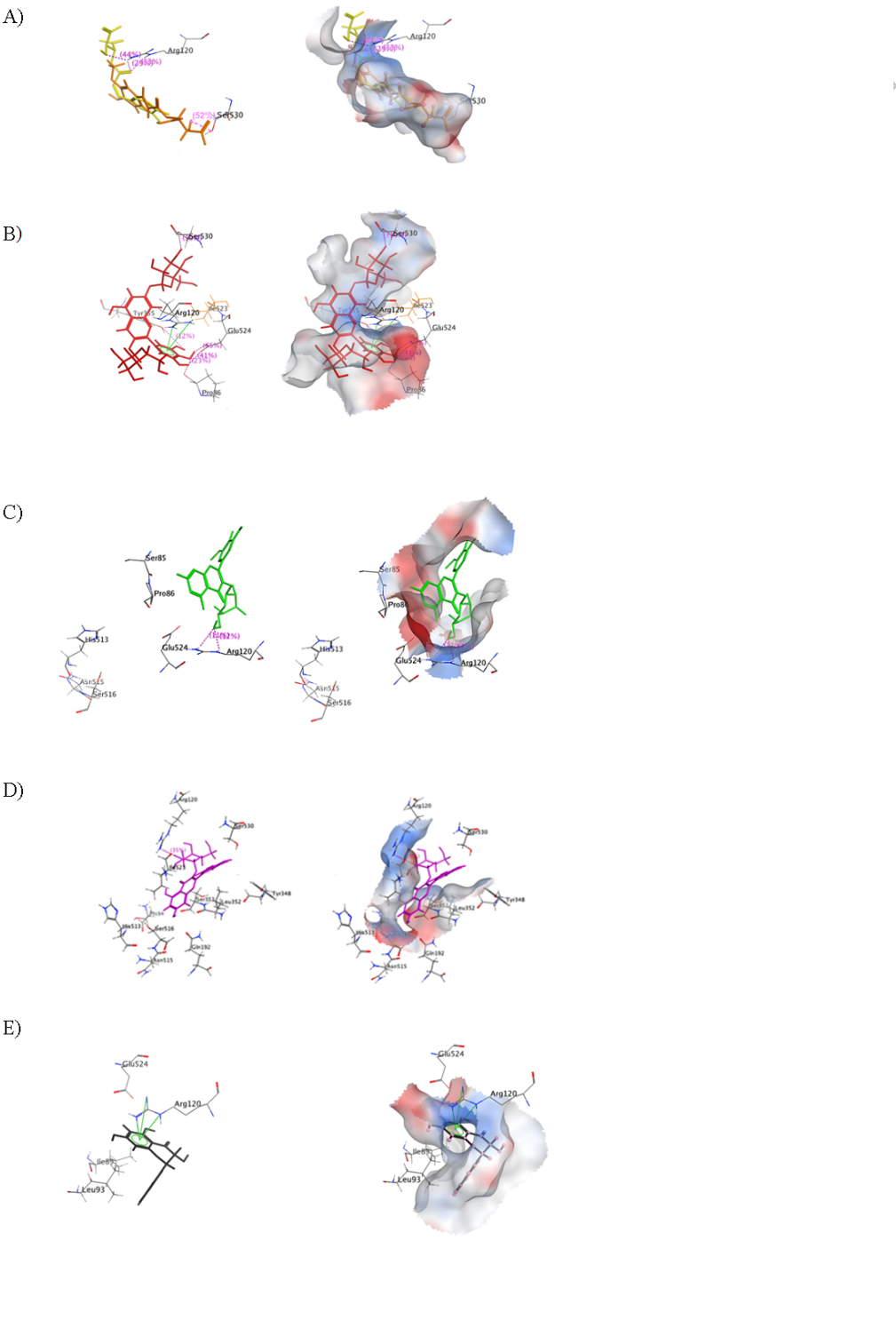


Table 1. Total Phenol Content (%GAE) and COX inhibition (%) of 19 samples of propolis extracts (P) and grape pomace (G). Samples are named as: 159 (Propolis extract 2010), 161 (Grape pomace extract 2010), 304 (Grape pomace Tannat 2013), 305 (Grape pomace Cabernet Sauvignon 2013), 306-309 (four 2013 Propolis samples), 310-315 (Propolis 2010 samples number 2, 9, 19, 50, 6 and 49 re-extracted in 2013), 316-319 (mixtures of propolis extract 306-309 with grape extract 304) and 333 (Grape pomace Tannat 2013).

Samples	**GAE (%)**	**DE**	**%inh COX1**	**DE**	**%inh COX2**	**DE**
(P) 159	5.4	0.3	**39.4**	**1.2**	**67.8**	**6.3**
(P) 161	5.7	0.5	**70.9**	**1.4**	**47.1**	**1.1**
(G) 304	7.6	1.2	---	---	**66.8**	**6.9**
(G) 305	3.9	0.9	---	---	**40.4**	**0.8**
(P) 306	4.0	0.2	**55.5**	**0.0**	**76.8**	**1.9**
(P) 307	5.6	0.1	**71.6**	**1.1**	**56.9**	**0.6**
(P) 308	8.1	0.1	**56.1**	**3.3**	**77.1**	**0.5**
(P) 309	9.7	0.5	**72.7**	**0.4**	**56.4**	**10.8**
(P) 310	12.4	1.1	**52.3**	**0.3**	**68.5**	**2.5**
(P) 311	12.7	1.0	**63.7**	**2.7**	**66.8**	**0.2**
(P) 312	11.2	0.2	**56.0**	**0.6**	**24.7**	**4.6**
(P) 313	15.0	1.7	**67.3**	**2.4**	**65.0**	**2.4**
(P) 314	12.9	0.3	**58.6**	**0.3**	**31.9**	**0.9**
(P) 315	9.6	0.6	**64.6**	**1.0**	**55.4**	**0.3**
(G+P) 316	6.4	0.2	---	---	**62.1**	**0.8**
(G+P) 317	7.2	1.0	---	---	**47.7**	**11.2**
(G+P) 318	6.4	0.6	**67.4**	**7.8**	**76.8**	**7.7**

(G+P) 319	3.1	1.0	---	---	**63.4**	**4.2**
(G) 333	3.3	0.5	---	---	**66.5**	**5.4**

Table 2. Analysis of contacting residues in COX-1 and COX-2 within 4.5 Å from Celecoxib. In the case of residue substitution, the order is COX-2/COX-1. Color codes: blue: identical residues making contacts in both enzymes; cyan: conserved residues making contacts in both enzymes; red: non conserved residues making contacts in both enzymes; yellow: identical residues making contacts only in COX-1; green: non conserved residues making contacts only in COX-1; pink: conserved residues making contacts only in COX-2.

Residues	IDENTICAL	CONSERVED	CONTACTING COX1	CONTACTING COX2
His58	YES		YES	YES
Thr 63	YES		YES	NO
Val85	YES		YES	YES
Arg89	YES		YES	YES
Gln161	YES		YES	YES
Tyr317	YES		YES	NO
Val318	YES		YES	YES
Leu321	YES		YES	YES
Ser322	YES		YES	YES
Tyr324	YES		YES	YES
Leu328	YES		YES	YES
Phe350	YES		YES	YES
Leu353	YES		YES	YES
Tyr354	YES		YES	YES
Trp356	YES		YES	YES
His/Arg482	NO	YES	NO	YES
Asn/Asp484	NO	NO	YES	NO
Ser/Ala485	NO	NO	YES	YES
Ile486	YES	YES	YES	YES
Phe487	YES	YES	YES	YES
Met491	YES	YES	YES	YES
Ile/Val492	NO	YES	YES	YES
Gly495	YES	YES	YES	YES
Ala496	YES	YES	YES	YES
Ser499	YES	YES	YES	YES
Leu500	YES	YES	YES	YES

Table 3. Results of the docking of Celecoxib at the binding site of COX-2 with different placement methods. First column: the number of the site identified in the Site Finder procedure. Second column: placement method used. Third column: root mean squares deviations of the docked ligand orientation with respect to the crystallographic coordinates. The selected placement method is highlighted in yellow.

Site number	Placement	RMSD	Score (KJ)
4	Alpha PMI	4.972	-39.712
6	Alpha PMI	1.269	-40.865
4	Alpha Triangle	1.243	-41.082
6	Alpha Triangle	0.712	-38.950
4	Proxy Triangle	5.127	-38.343
6	Proxy Triangle	5.152	-39.443
4	Triangle Matcher	5.127	-38.353
6	Triangle Matcher	5.074	-39.427

Table 4. Comparison of COX-2 docking score values and experimental binding free energies[3] for Celecoxib and the phenols: luteolin, myricetin and quercetin.

Molecule	**IC50** (μM)	Δ**G***bind* **(KJ/mol)**	**Docking score (KJ/mol)**
celecoxib[1]	0.49	-35.9963	-38.9364
luteolin[2]	57.2	-24.2033	-33.2636
myricetin[2]	200	-21.1020	-31.2919
quercetin[2]	200	-21.1020	-30.8918

[1] Badieyan S. Z, et al 2012.

[2] Ya-Di Li et al 2011.

[3] Experimental binding free energies are calculated from IC50 using the following relationship: Δ*G*bind = RTlnIC50, where *R* is ideal gas constant 8,31 x 10-3 KJ/mol and T is temperature in *K* (298 K is used), **(Zhong H et al, 2013).**

Table 5. Selection of molecules docked in chain A of both COX-1 and COX-2 ordered by their selectivity with respect to COX-2. First column: a PubChem code as a compound identifier (CID) was annotated in parentheses. Second column: phenols found in propolis and grapes were annotated by a "P" and a "G". Third column: the name of the basic structural scaffold. PH AC: Phenolic acids; A

NTHO: Anthocyanines; FLAV: Flavonoid; O-Glyc FLAV: glycosylatd flavonoid; O-Gal-FLAV; galactosidated flavnonoid; Fourth column: Lip: Lipinsky index; Fifth column: octanol/water partition coefficient; Sixth column: the number of hydrogen bonds (HB). Last three columns: best

Name molecule	Origin	Scaffold	Lip	logP(o/w)	HB	COX-1(A)	COX-2(A)	SI
(Z)-fertaric acid (72551456)	G	PH AC	1	0.97	2	-18.38	-35.65	1.94
Syringetin-3-O-glucoside (44259492)	G	O-Glyc FLAV	0	0.02	3	-24.48	-44.66	1.82
Delphinidin-3,7-diglucoside (44256889)	G	ANTHO	0	-1.86	6	-26.95	-44.15	1.64
(E)-fertaric acid (22298372)	G	PH AC	1	0.97	4	-24.34	-38.47	1.58
Malvidin-3-O-(6-acetyl)-glucoside (44256986)	G	ANTHO	0	1.53	2	-29.59	-45.51	1.54
Pinobanksin (73202)	P	FLAV	1	2.03	1	-16.73	-25.50	1.52
Laricitrin-3-O-glucoside	G	O-Glyc FLAV	0	-0.24	5	-25.61	-37.36	1.46
Malvidin-3,7-diglucoside (44256982)	G	ANTHO	0	-1.33	5	-27.98	-40.48	1.45
Petunidin-3-O-(6-acetyl)-glucoside (44256961)	G	ANTHO	0	1.26	4	-29.08	-41.84	1.44
Myricetin-3-O-galactoside (5491408)	P	O-Gal-FLAV	1	-0.51	7	-32.10	-46.09	1.44

docking scores in COX-1 and COX-2 and COX-2/COX-1 ratio as a selectivity index (SI). All scores are in $KJmol^{-1}$.

Table 6. Analysis of non bonded contacts between selected phenols with highest SI index and contacting residues in COX-2/COX-1. By default, hydrogen bond donor-acceptor distances (in Å) were annotated. Distances were measured between the closer acceptor atom of the H bond. Other type of contacts were registered as hydrophobic-aromatic (**Hyd-Aro**), hydrophobic (**Hyd**) or electrostatic (**E**).**First column**: contacting residues in COX-2/ COX-1. **All other columns**: distances between the selected phenols and residues in COX-2 and COX-1 (in parentheses). **First row: selected phenols and Celecoxib. Second row**: docking scores (COX-2/COX-1) and SI index. **Green cells** correspond to close contacts with COX-2 and weak or non existent contacts with COX-1. **White cells** correspond to weak (farther than 4.5 Å) or non-existent contacts with COX-2 and/or close contacts with COX-1. **Yellow cells** correspond to strong contacts with both enzymes. * Multiple contacts are separated by slashes.

	Z-fertaric acid	Syringetin-3-O-glucoside	Delphinidin-3,7-diglucoside	E-fertaric acid	Malvidin-3-O-(6-acetyl)-glucoside	Pinobanksin	Laricitrin-3-O-glucoside	Malvidin-3,7-diglucoside	Petunidin-3-O-(6-acetyl)-glucoside	Myricetin-3-O-galactoside	Celecoxib	SEL	NO SEL
	-35.65/-18.38 1.94	-44.66/-24.48 1.84	-44.15/26.95 1.64	-38.47/-24.34 1.58	-45.51/-29.59 1.54	-25.50/-16.73 1.52	-37.36/-25.61 1.46	-40.48/-27.98 1.45	-41.84/-29.08 1.44	-46.09/-32.10 1.44	-38.94/-41.61 0.94		
R106/R120	—(2.7/2.8/2.63*)	2.68/3.63(2.84/2.99)	2.86(E 2.46)	—(2.58/3.31)	2.76(2.62)	—-(3.24)	2.5/2.66(2.96)	2.89(—-)	2.93(2.87)	2.93(2.91)	E 3.37 (—-)	2	6
Q178/Q192	2.84(—)	3.21(—)	3.32(—)	3.00(---)	3.70(3.73)	---(—)	3.4(---)	---(---)	3.89(—)	3.89(3.49)	3.29(2.33)	6	3
L338/L352	2.96(—)	3.0(---)	—-(—)	2.76(—)	3.49(3.12)	3.20(---)	4.9(---)	3.20(—)	3.60(---)	2.74(3.86)	3.10(2.23)	6	3
S339/S353	2.32(—)	—-(—)	Hyd-Aro (—)	2.43(—)	3.46(2.99)	---(---)	3.2(---)	2.26(—)	Hyd-Aro (—)	Hyd-Aro(3.73)	3.06(—)	7	2
Y341/Y355	3.28(3.67)	2.73(—)	2.51/Hyd-Aro(—)	3.14(—-)	2.66/2.78(E 2.91)	—(3.50)	2.51(—-)	2.6(—-)	4.16(3.38)	3.67(2.70)	2.45(—-)	5	5
Y371/ Y385	---(---)	3.51(—)	4.07(2.81)	2.6(2.93)	2.76(2.75)	3.75(---)	3.47(—)	2.82(—)	2.77(—)	2.77(4.02)	3.42(Hyd-Aro)	4	6
R499/H513	2.55(—)	3.11(---)	3.15(---)	2.83(—)	2.75(—)	---(---)	3.18(—)	3.54(—)	3.01(---)	3.01(—)	3.18(—-)	10	0

A502/S516	**---(---)**	**---(---)**	**--(---)**	**2.99(—)**	**2.95(3.14)**	**---(---)**	**3.35(—)**	**---(---)**	**2.99(—)**	**2.99(3.89)**	**2.88(4.26)**	**3**	**2**
V509/I523	**---(---)**	**—-(—)**	**—(4.5)**	**3.12(—-)**	**2.61(4.35)**	**4.48(—-)**	**2.94(—)**	**2.40(—-)**	**3.04(—-)**	**4.27(Hyd-Aro)**	**3.33(3.0/Hyd)**	**5**	**3**
S516/S530	**2.63(—)**	**-—(—)**	**2.59(2.94)**	**3.67(2.78)**	**2.99(3.25)**	**2.2(—)**	**3.0(---)**	**---(---)**	**3.0(---)**	**3.2(3.1)**	**3.4/Hyd (4.14/Hyd)**	**4**	**4**

SUPPLEMENTARY MATERIAL A.. Selection of molecules docked in COX-1 for each COX-1 and COX-1 chains A PubChem code as a compound identifier (CID) was annotated in parentheses. The compounds with (*CID) refers to a CID parent code.

Second column: phenols found in propolis and grapes were annotated by a “P” and a “G” respectively. **Third column**: The name of the basic structural scaffold. PH AC: Phenolic acids and esthers; PROCY: Procyanidines; ANTHO: Anthocyanines; FLAV: Flavonoid; O-GlycFLAV: Flavonoid glycosylated in the O(C3) position: STYL: Stylbene; CAT: Catechin; **Fourth column: Lip:** Lipinski druglikeness. **Last three columns:** best scores of docking in 3KK6 for COX1 (chain A) and in 3LN1 for COX 2 (chain A) and the ratio or Selectivity Index SI. All scores are in **KJmol^{-1}.**

Name molecule	Origin	Scaffold	Lip	logP(o/w)	COX-1(A)	COX-2(A)	SI
(Z)-fertaric acid (72551456)	G	PH AC	1	0,974	-18.3768	-35.6540	1.94
Syringetin-3-O-glucoside (44259492)	G	O-Glyc FLAV	0	0.023	-24.4767	-44.6610	1.83
Delphinidin-3,7-diglucoside (44256889)	G	ANTHO	0	-1,856	-26.9476	-44.1491	1.64
(E)-fertaric acid (22298372)	G	PH AC	1	0,974	-24.3389	-38.4669	1.58
Malvidin-3-O-(6-acetyl)-glucoside (44256986)	G	ANTHO	0	1,525	-29.5909	-45.5130	1.54
Pinobanksin (73202)	P	FLAV	1	2,027	-16.7337	-25.4972	1.52

Laricitrin-3-O-glucoside	G	O-Glyc FLAV	0	-0.241	-25.6095	-37.3564	1.46
Malvidin-3,7-diglucoside (44256982)	G	ANTHO	0	-1,328	-27.9790	-40.4752	1.45
Petunidin-3-O-(6-acetyl)-glucoside (44256961)	G	ANTHO	0	1,261	-29.0842	-41.8395	1.44
Myricetin-3-O-galactoside (5491408)	P	O-Glyc FLAV	1	-0.505	-32.1023	-46.0899	1.44
Malvidin-3-O-glucoside (443652)	G	ANTHO	0	0,936	-28.0450	-38.6026	1.38
(Z)-coutaric acid (72551452)	G	PH AC	1	0,983	-24.5113	-33.6780	1.37
Pinobanksin-3-O-2-methylbutyrate (636686)	P	FLAV	1	3,903	-28.0782	-37.9832	1.35

Pyruvic derivative of peonidin-3-O-glucoside (443654)	G	ANTHO	0	0.977	-30.7892	-41.1514	1.34
Vitisin B of malvidin-3-O-(6-acetyl)-glucoside (*71308302)	G	ANTHO	0	1,394	-32.4967	-43.0901	1.33
Delphinidin-3-O-(6-acetyl)-glucoside (15385440)	G	ANTHO	0	0,997	-30.5079	-40.3422	1.32
Peonidin-3-O-(6-(Z)-p-coumaroyl)-glucoside (44256849)	G	ANTHO	0	3,532	-32.9757	-43.3712	1.32
Petunidin-3-O-glucoside (443651)	G	ANTHO	0	0,672	-34.1217	-44.8203	1.31
Pyruvic derivative of cyanidin-3-O-glucoside (187081)	G	ANTHO	0	0.713	-33.8395	-43.4235	1.28

Cyanidin-3-O-glucoside (441667)	G	ANTHO	0	0,681	-34.0558	-43.4852	1.28
Pyruvic derivative of delphinidin-3-O-glucoside (165558)	G	ANTHO	0	0.44	-32.6712	-41.6297	1.27
Petunidin-3-O-glucoside-4-vinylcatechol (*443651)	G	ANTHO	0	2,285	-31.7513	-40.2286	1.27
Quercetin-7-O-neohesperidoside (*5280343)	G	O-Glyc FLAV	0	-1,361	-23.3979	-29.1893	1.25
(E)-resveratrol (445154)	P	STYL	1	3,697	-27.6542	-34.3107	1.24
Pyruvic derivative of petunidin-3-O-glucoside (443651)	G	ANTHO	0	0.704	-33.8690	-41.4814	1.22

Myricetin-3-O-glucoside (5486615)	P	FLAV	1	-0,232	-28.3497	-34.4863	1.22
Pyruvic derivative of peonidin-3-O-(6-acetyl)-glucoside (65084)	G	ANTHO	0	1,566	-34.6989	-41.9044	1.21
5-methoxypinobanksin (*147459)	P	FLAV	1	2,291	-21.4097	-25.6703	1.19
(+)-gallocatechin (65084)	G	CAT	1	1,706	-27.1656	-32.4961	1.19
(E)-coutaric acid (57517924)	G	PH AC	1	0,983	-28.1903	-33.7151	1.19
Procyanidin B3 (4R-8 (+)C(-)C) (146798)	G	PROCY	0	3,656	-29.1358	-34.7079	1.19

Pyruvic derivative of malvidin-3-O-glucoside (443652)	G	ANTHO	0	0,968	-32.7200	-38.9011	1.19
Naringin (442428)	P	FLAV	1	-1,013	-25.2168	-29.8846	1.19
Isorhamnetin-3-O-glucoside (5318645)	G	O-Glyc FLAV	0	0,032	-29.8725	-35.4004	1.19
Delphinidin-3-O-glucoside (443650)	G	ANTHO	0	0,408	-33.3334	-39.3725	1.18
Peonidin-3-O-(6-acetyl)-glucoside (44256847)	G	ANTHO	0	1,534	-29.1080	-34.1205	1.17
Pinobanksin-3-O-acetate (148556)	P	FLAV	1	2,616	-26.8709	-31.3055	1.17
Peonidin-3-O-glucoside-4-vinylphenol (*443654)	G	ANTHO	0	2,831	-35.4258	-41.0893	1.16

(Z)-caftaric acid (72551521)	G	PH AC	1	0,710	-23.1690	-26.8212	1.16
Peonidin-3-O-glucoside-4-vinylguaiacol (*443654)	G	ANTHO	0	2,822	-34.8170	-40.0851	1.15
5-phenylpenta-2,4-dienoic acid (4024465)	G	PH AC	1	2,992	-22.5457	-25.8778	1.15
(-)epigallocatechin (65064)	G	CAT	1	1,706	-28.8449	-33.0353	1.15
Pyruvic derivative of malvidin-3-O-(6-acetyl)-glucoside (*443652)	G	ANTHO	0	1,557	-38.2046	-43.6648	1.14
Hesperetin (72281)	P	FLAV	1	2,371	-26.3092	-30.0021	1.14

Quercetin-3-O-glucoside (54758678)	P	O-Glyc FLAV	0	-0,232	-31.8597	-36.0758	1.13
Cyanidin-3-O-(6-acetyl)-glucoside (44256831)	G	ANTHO	0	1.27	-34.9334	-39.2047	1.12
Procyanidin B7 (4R-6 (+)E(-)C) (474541)	G	PROCY	0	3,656	-34.3419	-38.4693	1.12
Quercetin-3-O-galactoside (5281643)	G	O-Glyc FLAV	0	-0,232	-32.2105	-36.0747	1.12
Petunidin-3-O-glucoside-4-vinylphenol (*443651)	G	ANTHO	0	2,558	-34.1269	-37.8380	1.11
Malvidin-3-O-(6-(Z)-p-coumaroyl)-glucoside (44256995)	G	ANTHO	0	3,523	-38.3724	-42.5284	1.11
(E)-caffeic acid (717531)	P	PH AC	1	1,767	-17.4924	-19.1502	1.09

(E)-caftaric acid (6440397)	G	PH AC	1	0,710	-30.6743	-33.4320	1.09
Malvidin-3-O-glucoside-4-vinylguaiacol (44257037)	G	ANTHO	0	2,813	-36.1953	-39.4018	1.09
Peonidin-3-O-glucoside (443654)	G	ANTHO	0	0,945	-40.6425	-44.1688	1.09
Malvidin-3-O-(6-(E)-caffeoyl)-glucoside (44256989)	G	ANTHO	0	3.25	-49.5825	-53.5301	1.08
3-methoxygalangin (5281946)	P	FLAV	1	2,877	-30.7125	-33.1213	1.08
Delphinidin-3-O-glucoside-4-vinylphenol (*443650)	G	ANTHO	0	2,294	-36.2565	-38.9485	1.07
(-)-epicatechin (72276)	G	CAT	1	1,979	-31.9620	-34.2759	1.07

5-methoxypinobanksin-3-O-pentanoate (147459)	G	FLAV	1	4,239	-32.5877	-34.9025	1.07
Delphinidin-3-O-(6-(Z)-p-coumaroyl)-glucoside (44256898)	G	ANTHO	0	2,995	-36.0589	-38.0699	1.06
(E)-ferulic acid (445858)	G	PH AC	1	2,031	-18.8644	-19.7118	1.04
Petunidin-3-O-(6-(Z)-p-coumaroyl)-glucoside (44256963)	G	ANTHO	0	3,259	-48.9601	-50.9010	1.04
Pinobanksin-3-O-isobutyrate (46886756)	G	FLAV	1	3,461	-35.3827	-36.7175	1.04
(E)-phenethylcaffeate (CAPE) (5881787)	P	PH AC	1	3,786	-33.0729	-34.0879	1.03
Pinobanksin-3-O-propionate (4686755)	P	FLAV	1	3,091	-34.5204	-35.3182	1.02

Malvidin-3-O-glucoside-4-vinylphenol (44257035)	G	ANTHO	0	2,822	-35.2151	-35.8536	1.02
Vitisin B of malvidin-3-O-(6-p-coumaroyl)-glucoside (71308302)	G	ANTHO	0	3,392	-46.0515	-46.8649	1.02
(Z)-isoprenyl-p-coumarate (*637542)	G	PH AC	1	2,997	-30.6837	-31.2061	1.02
(+)-catechin (9064)	G	CAT	1	1,979	-28.0711	-28.5182	1.02
(E)-p-coumaric acid (637542)	P	PH AC	1	2.04	-18.6863	-18.9490	1.01
Pyruvic derivative of delphinidin-3-O-(6-acetyl)-glucoside (15385440)	G	ANTHO	0	1,029	-36.4063	-36.7029	1.01
Gallic acid (370)	G	PH AC	1	0,850	-17.7430	-17.8591	1.01

Methylgallate (7428)	G	PH AC	1	0,993	-16.7030	-16.7824	1.00
Petunidin-3-O-(6-(E)-p-coumaroyl)-glucoside (176449)	G	ANTHO	0	3,259	-43.9845	-44.0911	1.00
Quercetin (5280343)	P	FLAV	1	2,032	-30.9046	-30.8918	0.99
Apigenin (5280443)	P	FLAV	1	2,534	-32.2509	-32.2366	0.99
Procyanidin B2 (4S-8 (-)E(+)C) (122738)	G	PROCY	0	3,656	-26.9980	-26.9570	0.99
Pinocembrin (68071)	P	FLAV	1	2,688	-31.0525	-30.7366	0.99
(E)-isoprenyl-p-coumarate (*637542)	P	PH AC	1	2,997	-26.5819	-26.2773	0.99

Delphinidin-3-O-(6-(E)-p-coumaroyl)-glucoside (15922818)	G	ANTHO	0	2,995	-36.7586	-36.2540	0.99
Fisetin (5281614)	P	FLAV	1	2,305	-31.7236	-31.2655	0.99
(Z)-isoprenylcaffeate (5281790)	G	PH AC	1	2,724	-28.0210	-27.5780	0.98
Galangin (5281616)	P	FLAV	1	2,613	-31.5423	-30.8912	0.98
3,4-dihydroxyvinylbencene (151398)	P	STYL	1	2,159	-19.7208	-19.2241	0.98
Chrysin (5281607)	P	FLAV	1	2,842	-33.0241	-31.9032	0.97
Oroxylin A (5320315)	G	ANTHO	1	2,757	-33.1366	-31.9011	0.96

3,4-dimethoxycaffeic acid (717531)	P	PH AC	1	2,047	-19.6975	-18.9237	0.96
5-methoxychrysin (*5281607)	P	FLAV	1	3,106	-34.7306	-33.2377	0.96
Kaempferol (5280863)	P	FLAV	1	2,305	-31.9027	-30.4536	0.95
(E)-isoprenylcaffeate (5281790)	P	PH AC	1	2,724	-28.3596	-27.0418	0.95
Luteolin (5280445)	P	FLAV	1	2,261	-35.2028	-33.2636	0.95
Cyanidin-3-O-(6-(E)-p-coumaroyl)-glucoside (5282067)	G	ANTHO	0	3,268	-40.0661	-37.8402	0.94
Techtochrysin (5281954)	P	FLAV	1	3,106	-32.1514	-30.1100	0.94

3-hydroxy-5-methoxy-2-phenyl-2,3-dihydrochromen-4-one (73201)	G	FLAV	1	2,525	-30.5662	-28.5958	0.94
(E)-bencylcaffeate (5919576)	P	PH AC	1	3,698	-34.6455	-31.9619	0.92
Gentisic acid (3469)	P	PH AC	1	1,123	-16.2387	-14.9405	0.92
(E)-cinnamylcaffeate (5281787)	P	PH AC	1	3,922	-34.1499	-31.1033	0.91
Petunidin-3,7-diglucoside (44256973)	G	ANTHO	0	-1,592	-45.2018	-41.0182	0.91
Myricetin (5281672)	P	FLAV	1	1,759	-34.5835	-31.2919	0.90
Malvidin-3-O-glucoside-4-vinyl(+)-catechin (71308233)	G	ANTHO	0	3,162	-46.2655	-41.2607	0.89

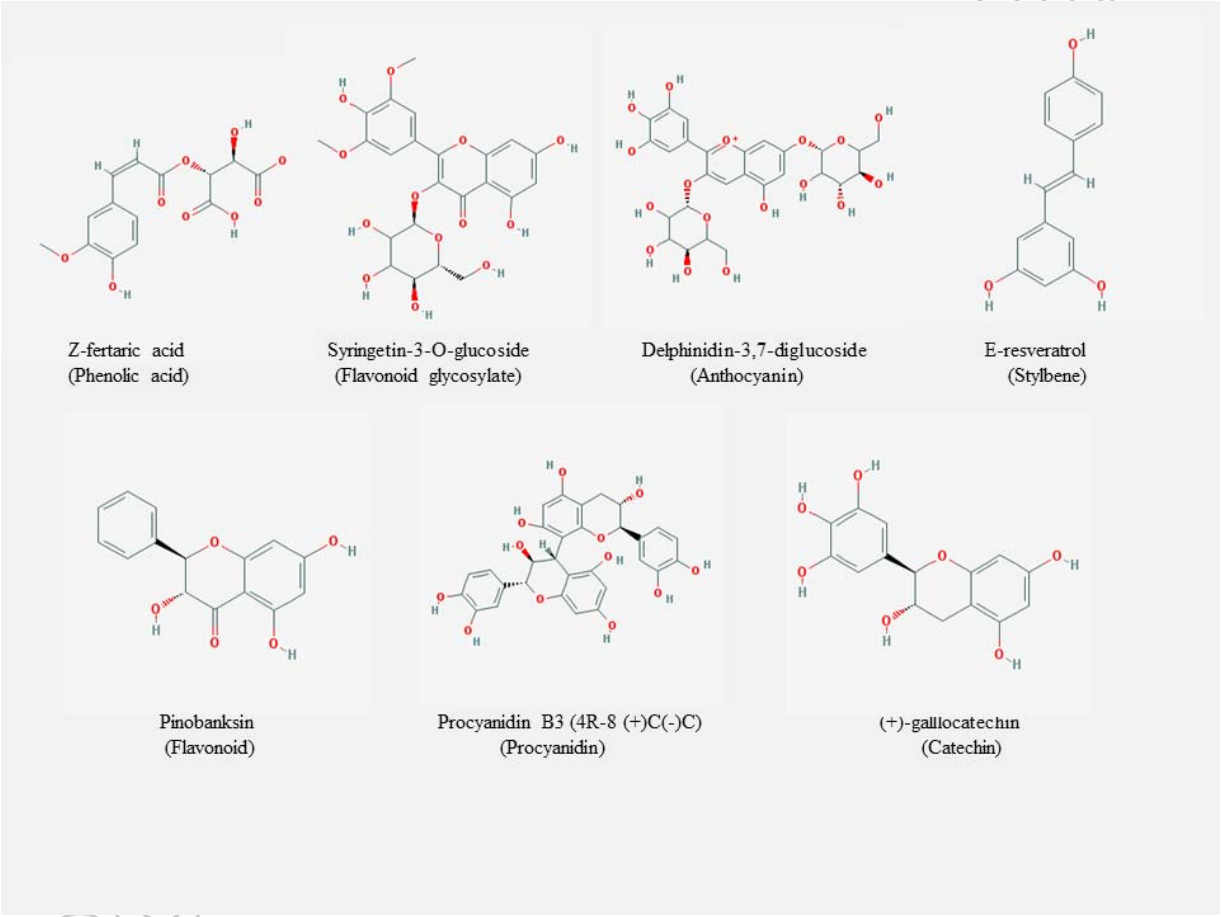
Pyruvic derivative of petunidin-3-O-(6-acetyl)-glucoside (44256961)	G	ANTHO	0	1,293	-41.3510	-36.5113	0.88
Pyruvic derivative of malvidin-3-O-(6-p-coumaroyl)-glucoside (72193651)	G	ANTHO	0	3,555	-46.5866	-40.4316	0.87
Cyanidin-3-O-(6-(Z)-p-coumaroyl)-glucoside (5282067)	G	ANTHO	0	3,268	-38.5589	-32.9773	0.86
Malvidin-3-O-(6-acetyl)-glucoside-4-vinylphenol	G	ANTHO	0	3,411	-37.4369	-31.8175	0.85
Protocatechuic acid (72)	G	PH AC	1	1,123	-19.7938	-16.7060	0.84
Pyruvic derivative of petunidin-3-O-(6-p-coumaroyl)-glucoside (72193651)	G	ANTHO	0	3,291	-43.4553	-35.1055	0.81

Procyanidin B5 (4S-6 (-)E(+)E) (124017)	G	PROCY	0	3,656	-32.7234	-25.8060	0.79
Peonidin-3-O-(6-(E)-p-coumaroyl)-glucoside (443654)	G	ANTHO	0	3,532	-43.0517	-31.7376	0.74
Procyanidin B1 (4S-8 (-)E(-)C) (11250133)	G	PROCY	0	3,656	-30.0618	-21.3864	0.71
Procyanidin B4 (4R-8 (+)C(+)E) (147299)	G	PROCY	0	3,656	-44.3546	-31.1426	0.70
Malvidin-3-O-(6-acetyl)-glucoside-4-vinylcatechol (*44257036)	G	ANTHO	0	3,138	-39.6112	-27.7391	0.70
Procyanidin B6 (4R-6 (+)C(-)C) (474540)	G	PROCY	0	3,656	-26.0772	-18.2565	0.70
Procyanidin B2-3-gallate (*122738)	G	PROCY	0	5,053	-34.2602	-22.5311	0.66

Malvidin-3-O-glucoside-4-vinyl-(-)-epicatechin (*44257035)	G	ANTHO	0	3,162	-42.5133	-23.1661	0.54
Pyruvic derivative of delphinidin-3-O-(6-p-coumaroyl)-glucoside (15922818)	G	ANTHO	0	3,027	-48.9244	-25.1664	0.51
Malvidin-3-O-glucoside-4-vinylcatechol (44257036)	G	ANTHO	0	2,549	-40.2287	-20.4051	0.51
Procyanidin B8 (4R-6 (+)C(+)E) (130556)	G	PROCY	0	3,656	-26.1243	-12.8295	0.49
Procyanidin B2 3'-gallate (*122738)	G	PROCY	0	5,053	-37.5729	-18.2800	0.49
Malvidin-3-O-(6-acetyl)-glucoside-4-vinylguaiacol (*44257037)	G	ANTHO	0	3,402	-37.3251	-16.8877	0.45

Malvidin-3-O-(6-(E)-p-coumaroyl)-glucoside-4-vinylphenol (*44256995)	G	ANTHO	0	5,409	-36.9565	-15.2519	0.41
Procyanidin C2 (4R-8 4R-8 (+)C(+)C(+)C) (11182062)	G	PROCY	0	5,333	-38.9112	-15.1028	0.39
Malvidin-3-O-(6-(E)-p-coumaroyl)-glucoside-4-vinylcatechol (*44256995)	G	ANTHO	0	5,136	-49.8263	-17.5521	0.35

SUPPLEMENTARY MATERIAL B. Molecular scaffold of basic structures annotated as PH AC: Phenolic acids; PROCY: Procyanidines; ANTHO: Anthocyanines; FLAV: Flavonoid; O-GlycFLAV: Flavonoid glysocylated in the O(C3) position: STYL: Stylbene and CAT: Catechin. Representative phenols were selected as examples.



SUPPLEMENTARY MATERIAL C: Superposition of COX-1 and COX-2 sequences and analysis of contacts with the selected phenols with highest SI scores and Celecoxib. **For each phenol, the top line is the sequence of COX-2 and the** bottom line is that of COX-1. ZFer: **Z-fertaric acid;** Syr: **Syringetin-3-O-glucoside;** Del: **Delphinidin-3,7-diglucoside;** EFer: **E-fertaric acid;** Mal: **Malvidin-3-O-(6-acetyl)-glucoside;** Pin: **Pinobanksin;** Lar: **Laricitrin-3-O-glucoside;** Mal2: **Malvidin-3,7-diglucoside;** Pet: **Petunidin-3-O-(6-acetyl)-glucoside;** Myr: **Myricetin-3-O-galactoside;** Cel: celecoxib. **In bold are highlighted the contact detected in a 4.5 A sphere around the phenol. NOTE: the numbering begins with the first residue and can not be matched with the number assigned in PDB crystals of COX-2 (3NL1) or COX-1 (3KK6).**

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PVNPCCYYPCQHQGICVRFGLDRYQCDCTRTGYSGPNCTIPEIWTWLRTTLRPS**P**SF**IH**FL**L**THGRWLWDFVNATFIRDT-
L**M**R**LVL**T**VR**SNLIPSPPTYNIAHDYISWESFSNVSYYTRI

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**xxxxxxxxx90xxxxxxxx500xxxxxxx10xxxxxxxx20xxxxxxxx30xxxxxxxx40xxxxxxxx50xxxxxxxx60xx
xxxxxx90xxxxxxxx80xxxxxxxx90xxxxxxxx600**

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PRPDAIFGETMVELGAPFSLKGLMGNPICSPQYWKPSTFGGEVGFKIINTASIQSLICNNVKGCPFTSFNVQD ZFERTARIC

CHPNSIFGESM**IE**M**G**APF**SL**KG**L**LGNPICSPEYWKASTFGGEVGFNLVKTATLKKLVCLNTKTCPYVSFHVPD

*****

P**R**PD**AIF**GET**MV**EL**GA**PF**SL**KG**L**MGNPICSPQYWKPSTFGGEVGFKIINTASIQSLICNNVKGCPFTSFNVQD
Syringetin-3-O-glucoside

CHPNSI**F**G**E**S**MIE**M**GA**PF**SL**KGLLGNPICSPEYWKASTFGGEVGFNLVKTATLKKLVCLNTKTCPYVSFHVPD

P**RPDAIF**GET**MVELGA**PF**SL**KGLMGNPICSPQYWKPSTFGGEVGFKIINTASIQSLICNNVKGCPFTSFNVQD
Delphinidin-3,7-diglucoside

CHPNSI**F**G**E**S**MIE**M**GA**PF**SL**KG**L**LGNPICSPEYWKASTFGGEVGFNLVKTATLKKLVCLNTKTCPYVSFHVPD

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P**R**PD**AIF**GET**MV**EL**GA**PF**S**LKGLMGNPICSPQYWKPSTFGGEVGFKIINTASIQSLICNNVKGCPFTSFNVQD EFERTARIC

CHPNSI**F**GES**MI**EM**GA**PF**SL**KGLLGNPICSPEYWKASTFGGEVGFNLVKTATLKKLVCLNTKTCPYVSFHVPD

P**R**PD**AIFG**ET**MVE**L**GA**PF**SL**KGLMGNPICSPQYWKPSTFGGEVGFKIINTASIQSLICNNVKGCPFTSFNVQD Malvidin-
3-O-(6-acetyl)-glucoside

CHPN**SIF**GES**MI**EM**GA**PF**SL**KG**L**LGNPICSPEYWKASTFGGEVGFNLVKTATLKKLVCLNTKTCPYVSFHVPD

P**R**PD**AIFG**ET**MVE**L**GA**PF**SL**KG**L**MGNPICSPQYWKPSTFGGEVGFKIINTASIQSLICNNVKGCPFTSFNVQD
Pinobanksin

CHPNSIFGESMI**E**MGAPFSLKGLLGNPICSPEYWKASTFGGEVGFNLVKTATLKKLVCLNTKTCPYVSFHVPD

P**R**PD**AIFG**ET**MVE**L**GA**PF**SL**KG**L**MGNPICSPQYWKPSTFGGEVGFKIINTASIQSLICNNVKGCPFTSFNVQD
Laricitrin-3-O-glucoside

CHPNSIFGESMI**E**MGAPFSLKGLLGNPICSPEYWKASTFGGEVGFNLVKTATLKKLVCLNTKTCPYVSFHVPD

P**R**PDAIFGETM**VE**LGA**P**FSLKGLMGNPICSPQYWKPSTFGGEVGFKIINTASIQSLICNNVKGCPFTSFNVQD Malvidin-
3,7-diglucoside

CHPNSIFG**E**SM**IE**MGAPFSLKGLLGNPICSPEYWKASTFGGEVGFNLVKTATLKKLVCLNTKTCPYVSFHVPD

P**R**PD**AIF**GET**MV**EL**GAP**F**SL**KG**L**MGNPICSPQYWKPSTFGGEVGFKIINTASIQSLICNNVKGCPFTSFNVQD
Petunidin-3-O-(6-acetyl)-glucoside

CHPNSIFG**E**SM**IE**MGA**P**FSLKGLLGNPICSPEYWKASTFGGEVGFNLVKTATLKKLVCLNTKTCPYVSFHVPD

PRPDAIFGETMVELGAPFSLKGLMGNPICSPQYWKPSTFGGEVGFKIINTASIQSLICNNVKGCPFTSFNVQD
Myricetin-3-O-galactoside

CHPN**SIF**GES**MI**EM**GA**PF**SL**KGLLGNPICSPEYWKASTFGGEVGFNLVKTATLKKLVCLNTKTCPYVSFHVPD

P**R**PD**AIF**GET**MV**EL**GA**PF**SL**KGLMGNPICSPQYWKPSTFGGEVGFKIINTASIQSLICNNVKGCPFTSFNVQD Celecoxib

CHP**NSIF**GES**MI**EM**GA**PF**SL**KGLLGNPICSPEYWKASTFGGEVGFNLVKTATLKKLVCLNTKTCPYVSFHVPD