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ANTIOXIDANT ACTIVITY OF *PIPER CANINUM* AND CYCLOOXYGENASE-2 INHIBITION BY METHOXYLATED FLAVONES

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Abstract

Background: This study investigated on antioxidant activity of *Piper caninum* and cyclooxygenase-2 inhibition by methoxylated flavones.

Materials and methods: The present study was carried out to quantify the total phenolic content and free radical scavenging activities of the crude extracts by Folin-Ciocalteu and 1,1-diphenyl-2-picrylhydrazyl free radical scavenging assay respectively.

Results: Methanolic extracts of *Piper caninum* exhibited the highest total phenolic content and free radical scavenging activities. All the pure compounds possessed significant cyclooxygenase-2 inhibition at physiological concentrations.

Conclusion: Based on *in vitro* and molecular docking, we therefore suggest that *Piper caninum* methoxylated flavones are potent inhibitors of cyclooxygenase-2 at physiological concentrations

Key words: *Piper caninum*; antioxidant; cyclooxygenase-2.

List of abbreviations: COX-2, cyclooxygenase-2; DPPH, 2,2-diphenyl-1-picrylhydrazyl; PGE₂, prostaglandin E2.

Introduction

There are 2000 species of Piperaceae distributed throughout the world, with one fifth located in Malaysia (de Waard and Anunciado, 1999). Piperaceae has been used by local people as an edible vegetable and treatment in traditional medicine with some species have been scientifically shown to possess anti-oxidant, anti-microbial and anti-inflammatory properties (Zakaria et al., 2010; Salleh et al., 2011; Rahmatullah et al., 2012; Thent et al., 2012). It is believed that this potent effect is due to the presence of polyphenolic compounds. Natural polyphenols have been shown to be beneficial to cardiovascular health as previously reported (Kawai, 2011; Kishimoto et al., 2013; Quiñones et al., 2013). However, most of the studies tended to use non-achievable *in vivo* bioavailable concentrations. The estimation of polyphenol levels in human plasma after the consumption of polyphenol-rich foods or beverages was reported around 0.1-1.0 μM (Manach et al., 2005; Nicholson et al., 2010). In particular to 5,7-dimethoxyflavone, the plasma concentration is on peak at 2.3 μM after 1 hour of oral administration (Walle, 2007).

COX-2 is an important mediator of inflammation which promoting pathogenesis and degenerative diseases. In activated monocyte or macrophages, COX-2 expression is upregulated, suggesting that inhibition of COX-2 may reduce pathogenesis through its anti-inflammatory effects (Linton and Fazio, 2004). The aim of this study was to evaluate the antioxidant activities of *P. caninum* crude extracts. Later, the isolated compounds from *P. caninum* were analyzed for cyclooxygenase-2 (COX-2) inhibition and *in silico* molecular docking.

Materials and Methods

General instrumentation

Melting points were determined using a Leica Gallen III hot stage melting point apparatus, and were uncorrected. Ultraviolet (UV) spectra were recorded on a Shimadzu UV-VIS 1601PC spectrophotometer in ethanol. Infrared (IR) spectra were recorded on a Perkin Elmer 1650 FTIR as a KBr disc or in Nujol. ¹H NMR spectra were recorded on a Bruker Avance apparatus at 400 MHz and ¹³C NMR spectra were measured at 100 MHz. Deuterated chloroform (CDCl₃) was used as the solvent. Mass spectra data were obtained from University College London, UK. Vacuum liquid chromatography was performed using Merck silica gel 230-400 mesh, while column chromatography was done on Merck silica gel 70-230 mesh. Thin layer chromatography aluminum sheets precoated with silica gel 60F₂₅₄ (0.2 mm thickness) were used to detect and monitor the components present in the crude extracts and fractions.

Solvent and chemicals

Gallic acid and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich Chemie (Steinheim, Germany).

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Analytical grade methanol (MeOH), chloroform (CHCl₃), ethyl acetate (EA), *n*-hexane and Folin-Ciocalteu's reagent were purchased from Merck (Darmstadt, Germany). Indomethacin was purchased from Cayman Chemical (Catalog No: 70270; Ann Arbor, MI, USA).

Plant materials

Sample of *P. caninum* was collected from Bau, Sarawak, Malaysia in July 2010. This species was identified by Mohizar Mohamad from Forest Research Centre, Kuching, Sarawak, Malaysia. The voucher specimen (UiTMKS3003) was deposited at Natural Product Research & Development Centre (NPRDC), UiTM Sarawak, Malaysia.

Extraction and purification of *P. caninum*

The dried stems (300 g) of *P. caninum* were ground into powder and extracted with *n*-hexane, ethyl acetate and MeOH sequentially by a Soxhlet extractor. Each of the extracts was filtered and concentrated under reduced pressure to yield the *n*-hexane (PCH) (7.12 g, 2.37%), ethyl acetate (PCEA) (9.24 g, 3.70%) and MeOH (PCM) (8.65 g, 3.46%) crude extracts. PCH (7.00 g) was fractionated by vacuum liquid chromatography and eluted with *n*-hexane, CHCl₃ and EtOAc in increasing polarity to afford six combined fractions (PCH1-6). Fractions PCH5 and PCH6 were combined and purified by column chromatography and further recrystallized using cold *n*-hexane to yield 5,7-dimethoxyflavone (**1**) as white crystalline needles (350.10 mg, 4.92%) and 4',5,7-trimethoxyflavone (**2**) as colorless crystalline needles (220.58 mg, 3.08%). The structure of compound (**1**) and (**2**) were determined and identified by previous data (Rajudin et al., 2010). Therefore, the structure was determined to be as described in Fig. 1 and Fig. 2.

5,7-Dimethoxyflavone (1): IR ν_{\max} (KBr) cm⁻¹: 2921 (C-H), 1643 (C=O), 1618 (C=C), 1603 (C=C), 1452 (C=C), 1121 (C-O); ¹H NMR δ_{H} (CDCl₃): 3.92 (3H, s, 5-OCH₃), 3.96 (3H, s, 7-OCH₃), 6.38 (1H, d, *J* = 2.1 Hz, H-8), 6.58 (1H, d, *J* = 2.1 Hz, H-6), 6.68 (1H, s, H-3), 7.50 (3H, m, H-3'/H-4'/H-5'), 7.87 (2H, dd, *J* = 2.1, 4.2 Hz, H-2'/H-6'); ¹³C NMR δ_{C} (CDCl₃): 55.7 (5-OCH₃), 56.4 (7-OCH₃), 92.9 (C-6), 96.2 (C-8), 109.1 (C-3), 109.3 (C-4a), 125.9 (C-2', C-6'), 128.9 (C-3'/C-5'), 131.2 (C-4'), 131.6 (C-1'), 159.9 (C-2), 160.6 (C-5), 160.9 (C-7), 164.1 (C-8a), 177.6 (C-4); EIMS *m/z* (M⁺) Calcd. for C₁₇H₁₄O₄: 282.

4',5,7-Trimethoxyflavone (2): IR ν_{\max} (KBr) cm⁻¹: 3019 (C-H), 2923 (C-H), 1642 (C=O), 1500 (C=C), 1567 (C=C), 1258 (C-O); ¹H NMR δ_{H} (CDCl₃): 0.89 (3H, s, 4'-OCH₃), 3.92 (3H, s, 7-OCH₃), 3.96 (3H, s, 5-OCH₃), 6.38 (1H, d, *J* = 2.4 Hz, H-8), 6.56 (1H, d, *J* = 2.4 Hz, H-6), 6.62 (1H, s, H-3), 7.02 (2H, d, *J* = 8.8 Hz, H-3'/H-5'), 7.84 (2H, d, *J* = 8.8 Hz, H-2'/H-6'); ¹³C NMR δ_{C} (CDCl₃): 8 55.5 (4'-OCH₃), 55.7 (7-OCH₃), 56.4 (5-OCH₃), 92.8 (C-6), 96.1 (C-8), 107.7 (C-3), 109.2 (C-4a), 114.4 (C-3'/C-5'), 123.9 (C-1'), 127.6 (C-2'/C-6'), 159.8 (C-4'), 160.7 (C-8a), 160.9 (C-5), 162.1 (C-2), 163.9 (C-7), 177.6 (C-4); EIMS *m/z* (M⁺) Calcd. for C₁₈H₁₆O₅: 312.

Determination of the total phenolic content (TPC)

The total phenolic content (TPC) was conducted using the Folin-Ciocalteu assay as previously described by Saucier and Waterhouse with minor modifications (Saucier and Waterhouse, 1999). The reaction mixture consisted of 20 μ L of sample solution, 1.58 mL of distilled water, 100 μ L of FC reagent (1 N) and 300 μ L of sodium carbonate solution 20% (w/v) to give a final volume of 2 mL. The mixture was incubated for 2 hours at room temperature and the absorbance was measured at 765 nm using a gallic acid (GA) calibration curve as the standard. All tests were carried out in triplicate.

Free radical scavenging activity measurements

The DPPH free radical scavenging calorimetric assay was carried out as described by Dasgupta and De with minor modifications (Dasgupta and De, 2004). DPPH (0.04% w/v) in methanol and sample solution were compared against the negative control containing reagents without the sample. MeOH was used as a blank. Absorbance at 517 nm was determined after 30 minutes of incubation in the dark at room temperature. All tests were carried out in triplicate. The percent inhibition activity (PI) was calculated as:

$$PI = [(A_0 - A_e) / A_0] \times 100$$

Where A_0 is the absorbance without the sample and A_e is the absorbance with the sample. Data are reported as the IC₅₀ value.

Molecular docking

Docking studies were carried out using AutoDock 4.2. The crystal structure of COX-2 with indomethacin (4COX.pdb) was taken from Brookhaven Protein Databank and indomethacin was deleted from the pdb file (Kurumbail et al., 1996). The three-dimensional structure of **1** [PubChem:88881], **2** [PubChem:79730], and indomethacin [PubChem:3715] were retrieved from PubChem (<http://pubchem.ncbi.nlm.nih.gov>). Missing hydrogens and Kollman partial charges were added before performing docking. The grid size was set to 90×90×90 points with 0.25 Å spacing centered on the COX-2 (Kongkathip et al., 2005). The default parameter setting was used to perform the docking calculations and only docking with the lowest binding energies were selected for further investigations. Interactions of the compounds with COX-2 were visualized and analyzed by Discovery Studio 4.0 (Accelrys Software Inc, San Diego, CA).

Cyclooxygenase-2 inhibition assay

The pure compounds from *P. caninum* were assayed using a commercial COX-inhibitor screening kit (Catalog No. 560131; Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. The reaction was measured

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spectrophotometrically at 415 nm after 30 minutes of incubation at room temperature, using indomethacin as a standard reference. A standard curve was constructed to determine the percentage of COX-2 inhibition. The IC₅₀ values were calculated from a non-linear regression plot of percent inhibition versus the logarithm of inhibitor concentration.

Statistical Analysis

The statistical analysis was performed by using SPSS 15.0 software (SPSS Inc. Chicago, IL, USA). Significant differences between the means were determined at a level of $p < 0.05$.

Results and Discussion

Total Phenolic Content (TPC) and DPPH free radical scavenging activity of *P. caninum* Extracts

As shown in Table 1, the TPC in different extraction solvents demonstrated an increasing trend from non-polar to polar. Phenolic compounds and individual extracts are considered the major contributors to the antioxidant activities of the *Piper* species (Nahak and Sahu, 2011).

In the context of extracts, the free radical scavenging capabilities may be a factor of the abundance of phenolic compounds in the extracts (see Table 1 and Table 2) (Amarowicz et al., 2004; Rathee et al., 2006). The highest activity of the MeOH extract may be due to the presence of polar phenolic compounds as compared to other extraction solvents.

From Table 1 and 2, the TPC and DPPH results indicated that the methanol extract possessed highest activity. The phenolic compounds in methanol extract were the main contributor to the free radical scavenging activity. However, the quantity of phenolic compounds as detected by the Folin-Ciocalteu assay does not totally reflect the quality of it in terms of free radical scavenging activity and antioxidant properties. In some cases, free radical scavenging activity is not solely due to phenolics but also dependent on the chemical structure of compounds present in the crude extracts (Liu et al., 2007). For instance, a pool of polyphenols carrying hydroxyl groups may dominate in one extract having greater antioxidant capacities when compared to another extract with similar pool of polyphenols without hydroxyl group. Moreover, the synergistic effect, i.e. the ability of several antioxidant compounds to reinforce each other, may contribute to the antioxidant activity (Kähkönen et al., 2001).

Table 1: Total phenolic content of *P. caninum* extracts.

Solvent extraction	Total phenolics
Hexane	ND
Ethyl acetate	51.73 ± 1.33
Methanol	108.43 ± 1.15

Data represent mean of triplicates ± S.E.M. Phenolics are expressed as mg of gallic acid equivalents/g of extract. ND denotes not detected.

Table 2: DPPH free radical scavenging activity of *P. caninum* extracts.

Sample	Solvent extraction	IC ₅₀ (µg/mL)
<i>P. caninum</i>	Hexane	ND
	Ethyl acetate	190
	Methanol	50
Ascorbic acid	-	2

Data represent concentration that provides 50% inhibition (IC₅₀) of three independent experiments. ND denotes not detected

Table 3: COX-2 inhibition effect of methoxylated flavones

Inhibitor	IC ₅₀ (µM)
1	1.73
2	1.58
Indomethacin	1.41

IC₅₀ values were calculated from a non linear regression plot of percent inhibition versus the logarithm of concentration. Indomethacin was used as a reference compound.

Table 4: Docking results in term of binding energy, inhibition constant, number of hydrogen bonds, amino acid interaction and bond lengths.

Ligand	Binding energy (kcal/mol)	Inhibition constant, K _i (nM)	No. of hydrogen bonds	Amino acid interaction	Bond Lengths [Å]
1	-8.90	300.49	1	Ser530	2.16
2	-9.62	88.77	2	His90 Ser530	1.87 2.17
Indomethacin	-10.23	31.86	2	Arg120 Tyr355	1.94 2.21

Ligand **1** and **2** are 5,7-dimethoxyflavone and 4',5,7-trimethoxyflavone respectively.

Table 5: Pi-Alkyl interaction between ligands and COX-2

Ligand	Amino acid interaction	Bond Lengths [Å]
1	Leu352	4.33
	Val523	4.71
2	Leu352	4.23
	Val523	4.77
Indomethacin	Tyr385	3.89
	Trp387	4.34
	Trp387	4.41
	Val349	4.75
	Val523	4.87
	Ala527	4.05
	Leu352	4.75

Ligand **1** and **2** are 5,7-dimethoxyflavone and 4',5,7-trimethoxyflavone respectively.

COX-2 inhibition activities of *P. caninum* pure compounds

In this study, we focused on COX-2 since it is an inducible enzyme in the presence of pro-inflammatory stimuli and plays an important role in the development of atherosclerosis and several inflammatory-related diseases (Cipollone and Fazia, 2006). In contrast COX-1 is constitutively express and does not involve in pathogenesis (Williams et al., 1999). The COX-2 inhibition by **1** and **2** were performed using indomethacin as a reference compound. This is the first report of *P. caninum* compounds on COX-2 inhibition at physiological concentrations. In general, all compounds significantly suppressed PGE₂ synthesis in a dose-dependent manner. Compound **2** (IC₅₀=1.58μM) showed higher inhibition than compound **1** (IC₅₀=1.73) (see Table 3). This suggest, the presence of methoxyl group at position 7- on the A ring and 4'- on the B ring, are important for COX-2 inhibition (see Figures 1 and 2). Similar observation was reported by Walle (2007) and Wu et al., (2002) where methoxyl group at position 7-on the A ring and 4'- on the B ring in other flavones having high activities respectively.

Molecular docking studies

In order to better understand the COX-2 inhibition by methoxylated flavones, molecular docking results were analyzed in Table 4. The molecular interaction between compounds and COX-2 were depicted in Figures. 3, 4 and 5.

As a reference compounds, indomethacin was docked into the active site of COX-2. The predicted binding mode of indomethacin with COX-2 (Fig. 3) was similar as previously reported (Kurumbail et al., 1996; Kurumbail et al., 2001). Binding energy between indomethacin and COX-2 active site is the lowest (see table 4) and therefore their interaction is the strongest among the compounds.

Compound **1** forms one hydrogen bond with the active site (Fig. 4). This bond was formed by Ser530 and 5-methoxyl group which is similar to diclofenac, a non-selective COX inhibitor. This Ser530 binding prevent arachidonic acid-COX interaction (Rowlinson et al., 2003).

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Compound **2** formed hydrogen bonds between Ser530 and His90 with 5-methoxyl and 4'-methoxyl group respectively (Fig.5). The His90 residue is known to be crucial for COX-2 activity since it forms a network of hydrogen bonds with other amino acids such as Arg120, Glu524 and Tyr355 which act as a gate to the COX binding site (Kurumbail et al., 2001; Rao and Knaus, 2008).

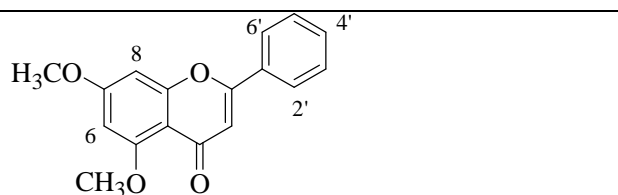


Figure 1: 5,7-Dimethoxyflavone (1)

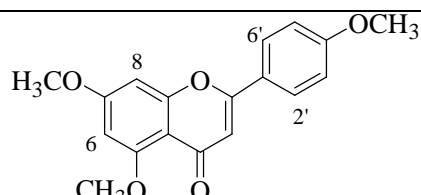


Figure 2: 4',5,7-Trimethoxyflavone (2)

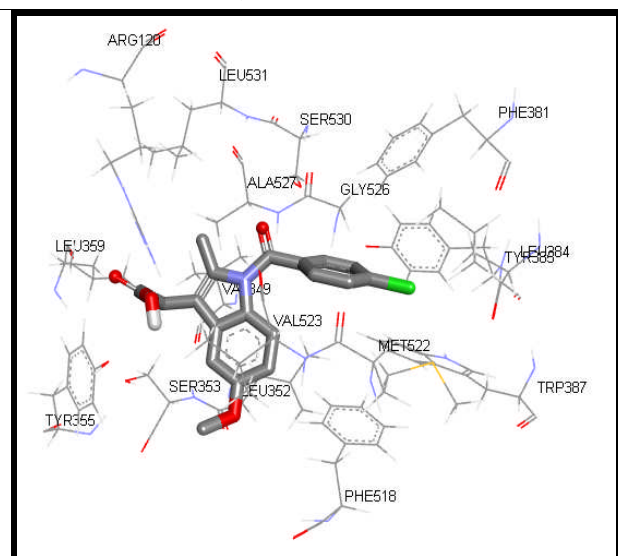


Figure 3: Binding of indomethacin into the active site of 4COX

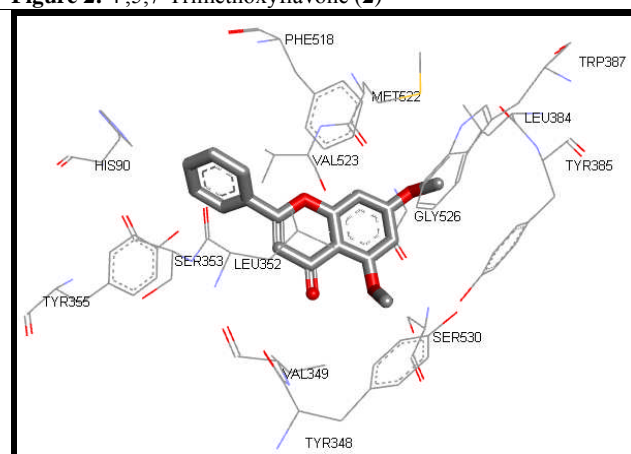


Figure 4: Binding of 1 into the active site of 4COX

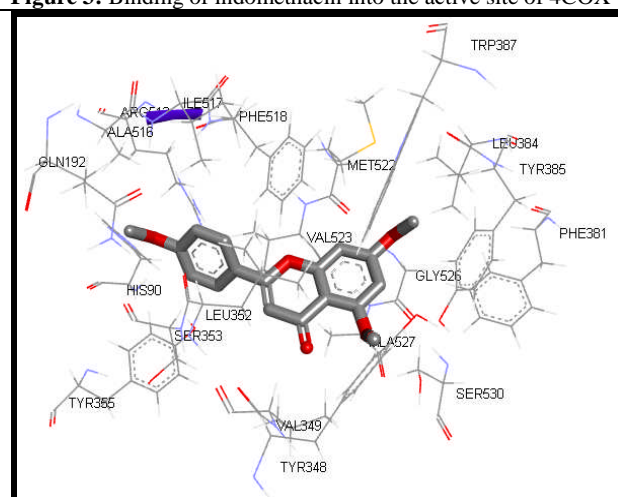


Figure 5: Binding of 2 into the active site of 4COX

Although compound **2** forms stronger hydrogen bond interactions with COX-2, indomethacin hydrophobic interaction particularly Pi-Alkyl together with hydrogen bond contributing stronger binding as a whole (see Table 4). Indomethacin formed 7 Pi-Alkyl interactions with amino acid residues Leu352, Val349, Val523, Ala527, Trp387 and Tyr385. In contrast, compound **2** showed only 2 Pi-Alkyl interactions with Val 523 and Leu352. Furthermore, in the context of bond length indomethacin interaction is better compared to compound **2** (Table 5). These interactions stabilize the binding of indomethacin to COX-2 active site. Thus, the lower binding energy observed in indomethacin than compound **2** is partly contributed by this hydrophobic interaction.

In conclusion, present study showed that *P. caninum* extracts possess antioxidant activities, with the methanolic extract being more potent than the other extracts. In addition, this study clearly demonstrated that the isolated compounds **1** and **2** inhibit COX-2 at physiological concentrations.

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