Journal of Research in Pharmacy

Research Article

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Novel piperazine substituted indole derivatives: Synthesis, anti-inflammatory and antioxidant activities and molecular docking

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Received: 07 November 2019 / Revised: 02 April 2020/ Accepted: 09 April 2020

ABSTRACT: In this work, a series of piperazine substituted indole derivatives were synthesized and evaluated for their *in vitro* antioxidant and anti-inflammatory activities. The results of antioxidant activity showed that compounds 2 (81.63%) and 11 (85.63%) had comparable DPPH free radical scavenging activity to Vit E (88.6%). The *in vitro* anti-inflammatory assays indicated that most of the compounds had more higher anti-inflammatory activities than standart ASA. Docking results revealed that compound 11 possessing the strongest anti-inflammatory activities showed the H-bond interactions with the key residues of COX-2 active site. It suggested that the anti-inflammatory activity of the compounds might result from COX-2 inhibition. It will be verified with further enzyme inhibition assays.

KEYWORDS: Anti-inflammatory activity; antioxidant activity; COX-2; piperazine; indole.

1. INTRODUCTION

Inflammation is a cellular protective response against a various triggering factors such as infectious agents, foreign pathogens, free radicals, tissue damage and cellular injury. Exaggerated and prolonged inflammation which seriously threatens human health may lead to tissue damage and various chronic diseases such as atherosclerosis, rheumatoid arthritis, sepsis, psoriasis, prostatitis, alzheimer and cancer [1,2]. Many enzymes, reactive oxygen, nitrogen and chlorine species and chemical mediators are released during the infiltration of inflammatory cells, and oxidative stress is induced[3]. Production of reactive species could initiate inflammation by activating multiple pathways such as redox-sensitive transcription factors including nuclear factor-kappaB (NF-kB) and activator protein-1 (AP-1). The reactive oxygen species (ROS) also is involved in conversion of arachidonic acid into proinflammatory intermediates and prostaglandins through cyclooxygenase-1 (COX-1) and lipoxygenase (LOX) [4-6]. Several studies show that inflammation and oxidative stress are related with each other in the development of many chronic disease such as diabetic complications [7,8] cardiovascular [9] and neurodegenerative diseases, [10,11] alcoholic liver disease, [12] and chronic kidney disease [13,14].

Indole compounds are one of the most studied heterocyclic scaffolds in medicinal chemistry because of their wide range of bioactivities such as anti-inflammatory, anti-viral, anti-HIV, anti-depressant, anti-histaminic, anti-hypertensive, and anti-diabetic [15,16]. To date only a few indole-based anti-inflammatory agents have been reported. Indomethacin (Figure 1), approved by Food and Drug Administration (FDA) in 1965 as a non-selective inhibitor of COX-1 and COX-2. It is used to reduce fever, pain and swelling by inhibiting the production of prostaglandins [17,18]. Tenidap (Figure 1) was developed as a COX/5-LOX inhibitor which have cytokine modulating anti-inflammatory and anti-rheumatoid activity. But, it was rejected due to its liver and kidney toxicity [19]. Acemetacin (Figure 1), [20] a prodrug of indomethacin, and etodolac (Figure 1), [21] a selective COX-2 inhibitor, are used for treatment of osteoarthritis and rheumatoid arthritis. Besides the anti-inflammatory effects of indomethacin, etodolac and acemetacin, they also have ROS and the reactive nitrogen species (RNS) scavenging activity [22]. Significant indole compounds such as melatonin, [23] tryptophan, serotonin, indole alkaloids, indole-3-acetic acid [24] and other synthetic derivatives [25-32] show

How to cite this article: Altuntaş TG, Baydar A, Kılıç-Kurt Z, Acar C, Yılmaz-Sarıaltın S, Çoban T. Novel piperazine substituted indole derivatives: Synthesis, anti-inflammatory and antioxidant activities and molecular docking. J Res Pharm. 2020; 24(3): 350-360.

ISSN: 2630-6344

https://doi.org/10.35333/jrp.2020.157

antioxidant potential by ROS and RNS scavenge ability. On the basis of the underlying mechanism of several chronic diseases, development of compounds inhibiting both oxidative stress and inflammation might be considered as an efficient approach for treatment of many diseases.

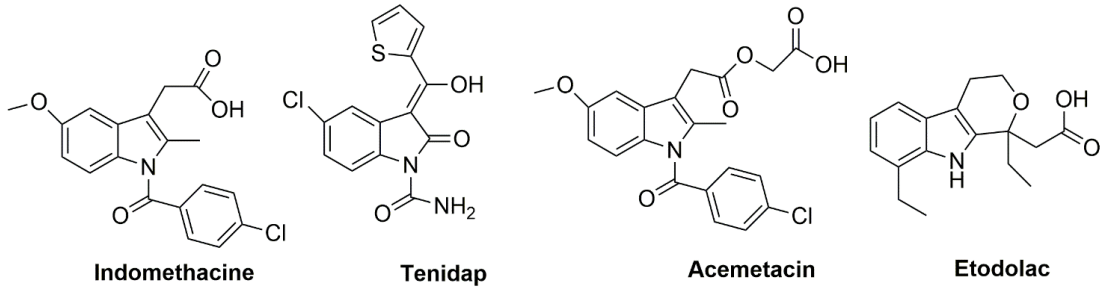


Figure 1. Indole based anti-inflammatory drugs.

We have previously reported the synthesis and *in vitro* antioxidant activity of a series of non substitue indole compounds bearing piperazine derivatives. They showed significant superoxide anion scavenging activity (88-69%) at 1 mM concentration [33]. In order to compare the activity, in this work, novel 5-methoxy and 5-fluoro indole derivatives (Figure 2) containing substituted-phenyl piperazine moiety at 2-position were synthesized. Their antioxidant and anti-inflammatory activity were evaluated. In addition, molecular docking of the most active compounds into COX-2 enzyme was performed by using Autodock vina.

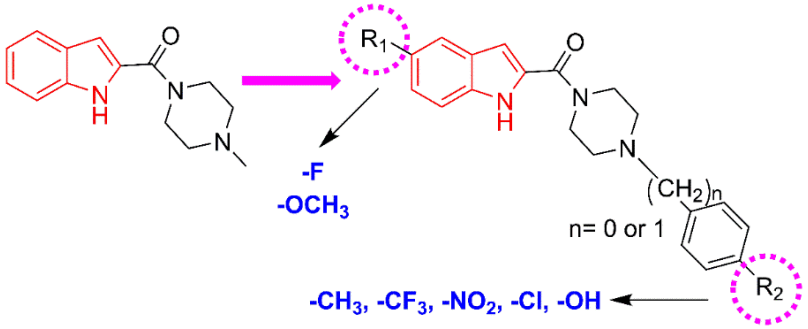


Figure 2. Designing of new 5-methoxy and 5-fluoro indole derivatives.

2. RESULT AND DISCUSSION

2.1. Chemistry

The synthesis of the target compounds containing substituted-piperazine derivatives is outlined in Figure 3. Compound **1-11** were obtained by reacting 5-substituted-indole-2-carboxylic acid with appropriate piperazine derivatives in the presence of carbonyldiimidazole (CDI) in anhydrous tetrahydrofuran (THF) under nitrogen atmosphere [34]. The structures of all synthesized compounds were characterized by ^{1}H NMR, ^{13}C NMR, MASS and elementel analysis. In the ^{1}H NMR spectra, the signal of the piperazine protons was observed as two broad singlet or triplet at around 2.41-3.61 ppm and 3.72-3.93 ppm for each molecule. NH proton of indole ring was observed as a characteristic singlet, which ranged from 11.38 to 11.72 ppm. H-3 proton of indole ring was detected between 6.67-6.84 ppm for each molecule. In the spectrum of the compounds **7** and **8**, a singlet at 3.49 and 3.50 ppm was observed due to the CH_2 protons, respectively.

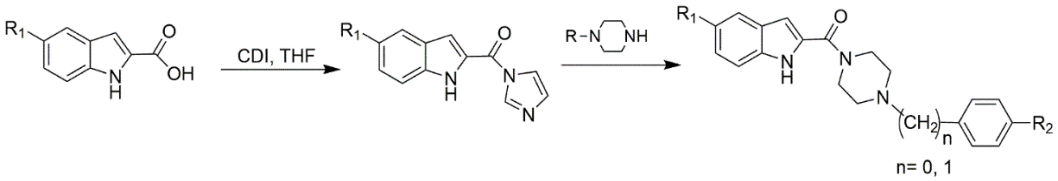


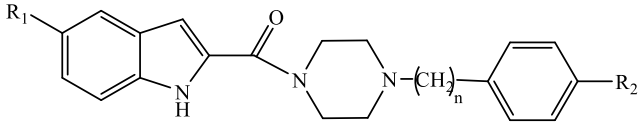
Figure 3. Synthesis of indole containing piperazine derivatives (**1-11**).

https://doi.org/10.35333/jrp.2020.157
J Res Pharm 2020; 24(3): 350-360

2.2. Biological activity

The target compounds were evaluated for their antioxidant activity by using different methodologies such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) stable radical and superoxide anion scavenging activity and lipid peroxidation (LP) inhibition. The obtained results were compared with the standard antioxidant vitamin E (Vit-E) in Table 1. Compounds **2** (81.63%) and **11** (85.63%) showed the highest DPPH free radical scavenging activity at 0.1 mM concentration, respectively. All compounds, except **2** and **11**, displayed no scavenging effect on LP and superoxide anion at 0.1 and 0.01 mM concentrations. Compound **2** had strong superoxide anion scavenging activity (51%) and comparable LP inhibition (46%) with vit-E (51%) at 0.1 mM. Better LP inhibition activity (79%) than vit-E (51%) was observed with compound **11** at 0.1 mM. Otherwise, compound **11** exhibited low superoxide anion scavenging activity (22%). Compounds **5, 8** and **10** showed no inhibition effect on all assay.

Table 1. Antioxidant activities of the newly synthesized compounds (**1-11**).



1-11

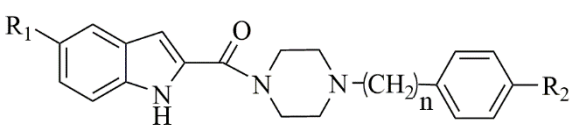
				DPPH Free Radical Scavenging Activity (%)[a,b,c]		Inhibition of LP (%)[a,b,c]		Superoxide anion scavenging activity (%)[a,b,c]	
Compd.	**R_1**	**R_2**	**n**	**0.1 mM**	**0.01 mM**	**0.1 mM**	**0.01 mM**	**0.1 mM**	**0.01 mM**
1	F	CH_3	0	14.63±0.43	2.88±0.04	NA	NA	NA	NA
2	OCH_3	CH_3	0	81.63±0.04	25±0.04	46±2	40±1	51±3	42±3
3	F	F	0	4.73±0.94	3.66±1.85	NA	NA	NA	NA
4	OCH_3	F	0	6.93±0.38	4.04±0.25	5±0.5	NA	NA	NA
5	F	CF_3	0	NA	NA	NA	NA	NA	NA
6	OCH_3	CF_3	0	2.62±0.38	NA	NA	NA	NA	NA
7	F	F	1	2.54±0.55	NA	NA	NA	NA	NA
8	OCH_3	F	1	NA	NA	NA	NA	NA	NA
9	OCH_3	Cl	0	2.15±0.94	NA	NA	NA	NA	NA
10	OCH_3	NO_2	0	NA	NA	NA	NA	NA	NA
11	F	OH	0	85.63±0.43	15.66±0.86	79±3	68±2	22±2	NA
Vit E				88.6±0.04	49.18±0.81	51±5	28±2	35±3	11±3

[a]The values represent the average of 3 determinations ± SD.
[b]Compounds were diluted with DMSO (solvent showed no antioxidant activity).
[c]$p < 0.05$ vs. DMSO by ANOVA/Tukey's test. NA; no activity.

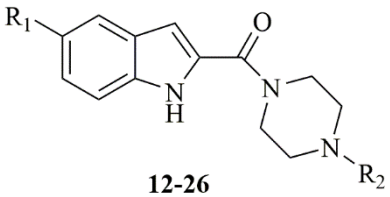
The human red blood cell (HRBC) membrane stabilization could be a significant *in vitro* measure of anti-inflammatory activity of the drugs. The membrane stabilizing activities of the newly synthesized compounds (**1-11**) and previously synthesized compounds (**12-26**) [33] were evaluated using heat induced human erythrocyte hemolysis in comparison with acetylsalisilic acid (**ASA**) used as reference drug. Table 2 depicted that 5-substituted indole derivatives (**1-11**, except compound **4**) showed more inhibition of heat induced hemolysis compared to indole derivatives (**12-26**) as well as **ASA**. Substitution at 5-position of the indole ring with fluoro and methoxy groups might have contributed to the increased anti-inflammatory potency of the compounds. Replacement of fluoro (**1**, IC_{50}=1.19 mM) with methoxy (**2**, IC_{50}= 0.68 mM) at 5-position of indole ring, resulted in 1.7-fold increase in membrane stabilizing activity. In contrast, the same methoxy substitution at 5-position of indole ring decreased the activity of compounds **4**, **6** and **8** relative to compound **3**, **5** and **7**. Replacing the hydroxyl group at 4'-position of phenyl piperazine moiety (**11**, IC_{50}= 0.33 mM) with fluoro (**3**, IC_{50}= 0.64 mM) and methyl (**1**, IC_{50}= 1.19 mM) caused a dramatic loss in membrane stabilization

activity, while the trifluoromethyl group (**5**, IC_{50}= 0.43 mM) is well tolerated. In case of 5-methoxy indole derivatives, the nitro group at 4′-position of phenyl piperazine exhibited stronger membrane stabilization activity (**10**, IC_{50}= 0.40 mM) than corresponding derivatives bearing chloro (**9**), CF_3 (**6**), fluoro (**4**) and methyl (**2**). The compounds **2** and **11** were found 2- and 4.3-fold more active than **ASA** as anti-inflammatory activity which showed the correlation with antioxidant activity of them. Although 5-methoxy substitution on indole ring resulted in 5.8-fold increase in membrane stabilization activity of compound **10** compared to corresponding indole derivative **21**, it did not lead to significantly change in membrane stabilization activity of the compounds **4** and **22**.

Table 2. Human red blood cell membrane stabilizing effects of the compounds.



1-11



12-26

Compd.[a]	R_1	R_2	n	IC_{50} ± SD (mM)	Compd.[a]	R_1	R_2	n	IC_{50} ± SD (mM)
1	F	CH_3	0	1.19±0.05*	**14**	H	3-nitro-2-pyridyl	0	2.21±0.03*
2	OCH_3	CH_3	0	0.68±0.01*	**15**	H	CH_3	0	1.95±0.01*
3	F	F	0	0.64±0.01*	**16**	H	CH_2CH_2OH	0	1.83±0.05*
4	OCH_3	F	0	3.11±0.02*	**17**	H	3-chlorobenzyl	0	2.54±0.01*
5	F	CF_3	0	0.43±0.01*	**18**	H	diphenylmethyl	0	2.76±0.06*
6	OCH_3	CF_3	0	0.54±0.02*	**19**	H	cyclohexyl	0	2.38±0.08*
7	F	F	1	0.46±0.04*	**20**	H	phenyl	0	5.45±0.09*
8	OCH_3	F	1	0.68±0.02*	**21**	H	*p*-nitrophenyl	0	2.34±0.03*
9	OCH_3	Cl	0	0.56±0.01*	**22**	H	*p*-fluorophenyl	0	3.84±0.02*
10	OCH_3	NO_2	0	0.40±0.03*	**23**	H	pyrimidine-2-yl	0	2.32±0.07*
11	F	OH	0	0.33±0.02*	**24**	H	acetyl	0	1.97±0.01*
12	H	CHO	0	2.32±0.01*	**25**	H	cyclopropylcarbonyl	0	1.73±0.02*
13	H	H	0	2.27±0.02*	**26**	H	ethoxycarbonyl	0	2.30±0.02*
ASA				1.42±0.03*					

[a] Synthesis of compound 12-26 was previously reported in lit. 33. (*) Statistically significant as compared to control. $p<0.05$.

2.3. Molecular docking and prediction of molecular properties

In order to understand whether the anti-inflammatory activity of the compounds is correlated with COX-2 (PDB code: 3NT1) enzyme inhibition, the molecular docking study was performed using Autodock vina. Firstly, the validation of the docking procedure was done by docking of crystallographic naproxen over 3NT1. The docked naproxen is superimposed on crystallographic naproxen forming two hydrogen bonds with Arg120 and one hydrogen bond with Tyr355 and RMSD value was found as 0.702 Å (Figure 4, left) [35]. Compound **11** possessing the best anti-inflammatory activity formed a hydrogen bond between amide carbonyl and NH of Arg120 at the distance of 2.23 Å (Figure 4, right). The docking results suggested that the anti-inflammatory activities of the compound **11** might correlate with its COX-2 interactions. In order to verify the results of molecular docking and present the mechanism of anti-inflammatory activity, further COX-2 enzyme inhibition studies will be performed.

Molecular properties of the synthesized compounds (**1–11**) were calculated using online Molinspiration property program [36]. The predicted volume, topological polar surface area (TPSA), number of violations and Lipinski parameters [37] such as molecular weight (MW), number of rotatable bonds (*n*ROTB), number of hydrogen bond acceptors (*n*ON) number of hydrogen bond donors (*n*OHNH), and lipophilicity (*mi*LogP) were calculated. As shown in Table 3, all compounds confirm the Lipinski's rules with logP values ranged from 2.11-4.35, MW ranging from 339.40-403.40, HBA value of ≤10, HBD value of ≤5 and *n*ROTB values of <10.

The percentage of absorption (%ABS) is calculated by using %ABS = 109 − (0.345 x TPSA) [38]. All compounds (except compound **10**) have the good ABS% value of >80%, suggesting that the synthesized compounds provide acceptable flexibility and favorable permeability and oral bioavailability.

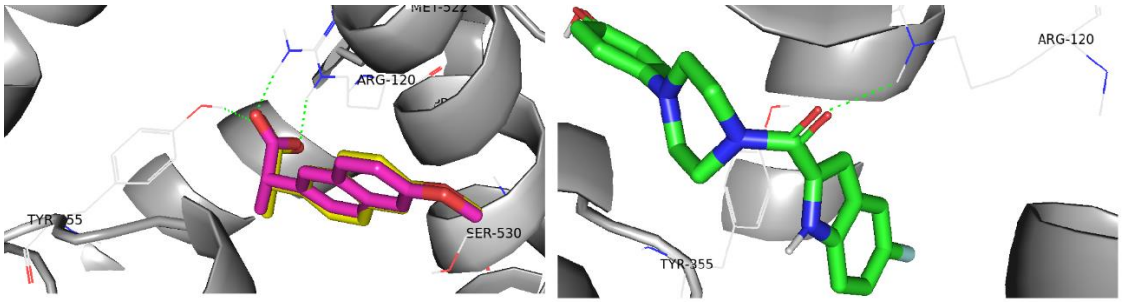


Figure 4. The superimposition of crystal naproxen (yellow) and docked naproxen (magenta) (left). The predicted binding mode of compound **11** (green) in the catalytic site of COX-2 (pdb code: 3NT1) are generated by the programme PyMOL (right). The hydrogen bonds are presented by dotted green lines and residues forming hydrogen bonds with ligand are shown in element coloured line.

Table 3. Prediction of molecular properties parameters and drug-likeness scores of indole containing piperazine derivatives (**1-11**).

Cpd	MW[a]	Volume	%ABS[b]	TPSA[c]	*n*ROTB[d]	*n*ON[e]	*n*OHNH[f]	LogP[g]	*n*violations
Rule	<500	-	-	-	<10	≤10	≤5	≤5	≤1
1	337.40	306.84	95.43	39.34	2	4	1	3.04	0
2	349.43	327.45	92.25	48.57	3	5	1	3.91	0
3	341.36	295.21	95.43	39.34	2	4	1	2.76	0
4	353.40	315.82	92.25	48.57	3	5	1	3.62	0
5	391.37	321.57	95.43	39.34	3	4	1	3.49	0
6	403.40	342.19	92.25	48.57	4	5	1	4.35	0
7	355.39	312.01	95.43	39.34	3	4	1	2.46	0
8	367.42	332.62	92.25	48.57	4	5	1	3.32	0
9	369.85	324.43	92.25	48.57	3	5	1	4.14	0
10	380.40	334.23	76.43	94.40	4	8	1	3.42	0
11	339.37	298.30	88.44	59.57	2	5	2	2.11	0

[a] MW: Molecular weight; [b] %ABS: Percentage absorption; [c]TPSA: Topological polar surface area; [d] *n*ROTB: Number of rotatable bonds; [e] *n*ON: Number of hydrogen acceptors; [f] *n*OHNH: Number of hydrogen donors; [g] LogP: Log octanol/water partition coefficient.

3. CONCLUSION

In conclusion, several 5-substituted indole derivatives bearing piperazine group were synthesized for evaluation of their antioxidant and anti-inflammatory activity based on our previously obtained results from corresponding non-substituted indole derivatives [33]. The *in vitro* antioxidant and anti-inflammatory activity results revealed that the 5-substituted indoles were more active than non-substituted indole derivatives, suggesting substitution at 5-position of indole ring could be important for activity. Compounds **2** and **11** possessing the highest DPPH radical scavenging activity also showed strong anti-inflammatory activity with IC_{50} value of 0.68 and 0.33 mM. Moreover, compound **11** located into active site of COX-2 forming hydrogen bond with Arg120. In the future, whether the strong anti-inflammatory activity of compound **11** result from COX-2 inhibition will be evaluated.

4. MATERIALS AND METHODS

The chemical reagents were purchased from commercial suppliers and used without further purification. The reactions were monitored and the purity of the products was checked by thin layer chromatography (TLC). Merck silica gel 60 F_{254} chromatoplates were used for TLC. Uncorrected melting points were measured on a Büchi B-540 capillary melting point apparatus. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on Varian Mercury 400 MHz FT spectrometer (Agilent Technologies, USA) in DMSO-

d_6, $CDCl_3$, and CD_3OD as solvent. The chemical shifts (δ) were recorded in parts per million relative to tetramethylsilane (TMS) and coupling constants (J) are reported in Hertz. The Mass spectra were recorded on a Waters micromass ZQ, using ESI(+). Elemental analysis were carried by Leco-932 CHNS-O analyzer. The results of the elemental analysis (C, H, N) were within ±0.4% of the calculated amounts. Biological assays were carried by using a microplate reader (SpectraMax 190, Molecular Devices, USA).

4.1. General procedure for the synthesis of compounds

A solution of CDI in THF (3 ml, 3.7 mmol) was added to 5-substituted-indole-2-carboxylic acid (3.1 mmol) in THF (5 ml) at room temperature and stirred for 1 h under N_2 atmosphere. Then, the reaction mixture was cooled to 0°C and N-substituted piperazine derivatives (3.7 mmol) in THF (3 ml) were added and stirred for further 17-18 h at room temperature. Basic work-up ($CHCl_3$, sat. $NaHCO_3$) was applied, evaporated under vacuo and recrystallization from ethyl acetate:*n*-hexane provided the desired compounds.

4.1.1. (5-Fluoro-1H-indol-2-yl)(4-(p-tolyl)piperazin-1-yl)methanone (1)

CAS Registry Number: 2325638-42-2. Yield: 62%; mp.: 233-234 °C. ^{1}H-NMR (400 MHz, DMSO-d_6); δ ppm: 2.19 (s, 3H, -CH_3), 3.14 (t, 4H, piperazine-H), 3.87 (bs, 4H, piperazine-H), 6.81 (d, 1H, J_m=1.6 Hz, H-3), 6.86 (d, 2H, J_o=8.8 Hz, H-2',6'), 7.01-7.07 (m, 3H), 7.36 (dd, 1H, J_m=2.8 Hz, J_o=10 Hz), 7.41 (dd, 1H, J_m=4.4 Hz, J_o=8.8 Hz), 11.71 (s, 1H, N-H). ^{13}C-NMR δ ppm (DMSO-d_6): 19.957; 49.043; 103.950; 105.386; 111.795; 113.155; 116.070; 126.787; 128.194; 129.352; 131.539; 132.583; 148.563; 155.893; 158.210; 161.593. MS (ESI +) m/z: 338.3 (M + H, 100%). Anal. Calcd for $C_{20}H_{20}FN_3O \cdot 0.1H_2O$: C, 70.81; H, 6.00; N, 12.38. Found: C, 70.84; H, 6.21; N, 12.31.

4.1.2. (5-Methoxy-1H-indol-2-yl)(4-(p-tolyl)piperazin-1-yl)methanone (2)

Yield: 53%; mp.: 203-205 °C. ^{1}H-NMR (400 MHz, DMSO-d_6); δ ppm: 2.25 (s, 3H, -CH_3), 3.20 (bs, 4H, piperazine-H), 3.80 (s, 3H, -OCH_3), 3.93 (bs, 4H, piperazine-H), 6.79 (s, 1H, H-3), 6.88-6.93 (m, 3H), 7.09-7.13 (m, 3H), 7.37 (d, 1H, J_o=8.8, H-7), 11.50 (s, 1H, N-H). ^{13}C-NMR δ ppm (DMSO-d_6): 20.038; 49.155; 55.228; 101.894; 103.845; 112.874; 114.307; 116.151; 127.086; 128.290; 129.433; 130.134; 131.186; 148.621; 153.741; 161.994. MS (ESI +) m/z: 350.5 (M + H, 100%). Anal. Calcd for $C_{21}H_{23}N_3O_2 \cdot 0.1H_2O$: C, 71.81; H, 6.65; N, 11.96. Found: C, 71.76; H, 6.69; N, 11.89.

4.1.3. (5-Fluoro-1H-indol-2-yl)(4-(4-fluorophenyl)piperazin-1-yl)methanone (3)

CAS Registry Number: 902025-79-0. Yield: 24%; mp.: 242-244 °C. ^{1}H-NMR (400 MHz, DMSO-d_6); δ ppm: 3.16 (t, 4H, piperazine-H), 3.88 (bs, 4H, piperazine-H), 6.82 (s, 1H, H-3), 6.97-7.10 (m, 5H), 7.37 (dd, 1H, J_m=2.4 Hz, J_o=9.6 Hz), 7.42 (dd, 1H, J_m=4.4 Hz, J_o=8.8 Hz), 11.72 (s, 1H, N-H).^{13}C-NMR δ ppm (DMSO-d_6): 49.376; 104.054; 105. 467; 111.895; 113.240; 115.366; 117.690; 126.853; 131.574; 132.664; 147.614; 155.128; 155.974; 157.475; 158.283; 161.674. MS (ESI +) m/z: 342.2 (M + H, 100%). Anal. Calcd for $C_{19}H_{17}F_2N_3O$: C, 66.85; H, 5.01; N, 12.30. Found: C, 66.62; H, 5.21; N, 12.20.

4.1.4. (4-(4-Fluorophenyl)piperazin-1-yl)(5-methoxy-1H-indol-2-yl)methanone (4)

CAS Registry Number: 878987-70-3. Yield: 28%; mp.: 172-173 °C. ^{1}H-NMR (400 MHz, DMSO-d_6); δ ppm: 3.15 (t, 4H, piperazine-H), 3.74 (s, 3H, -OCH_3), 3.88 (bs, 4H, piperazine-H), 6.73 (s, 1H, H-3), 6.84 (dd, 1H, J_m=2.4 Hz, J_o=8.8 Hz, H-6), 6.96-7.00 (m, 2H), 7.05-7.09 (m, 3H), 7.31 (d, 1H, J_o=8.8 Hz), 11.45 (s, 1H, N-H). ^{13}C-NMR δ ppm (DMSO-d_6): 49.391; 55.220; 101.894; 103.867; 112.874; 114.330; 115.358; 117.667; 127.078; 130.096; 131.193; 147.645; 153.749; 155.113; 157.452; 161.994. MS (ESI +) m/z: 354.2 (M + H, 100%). Anal. Calcd for $C_{20}H_{20}FN_3O_2 \cdot 0.2H_2O$: C, 67.28; H, 5.75; N, 11.77. Found: C, 67.12; H, 5.79; N, 11.71.

4.1.5. *(5-Fluoro-1H-indol-2-yl)(4-(4-(trifluoromethyl)phenyl)piperazin-1-yl)methanone (5)*

Yield: 24%; mp.: 297-298 °C. ^{1}H-NMR (400 MHz, DMSO-d_6); δ ppm: 3.43 (t, 4H, piperazine-H), 3.90 (bs, 4H, piperazine-H), 6.84 (s, 1H, H-3), 7.04 (dd, 1H, J_o=9.2 Hz, J_m=2.4 Hz), 7.08 (d, 2H, J_o=8.4 Hz, H-2',6'), 7,37 (dd, 1H, J_m=2.8 Hz, J_o=9.6 Hz) 7.43 (dd, 1H, J_m=4.8 Hz, J_o=9.2 Hz), 7.53 (d, 2H, J_o=8.8 Hz, H-3',5'), 11.69 (s, 1H, N-H). ^{13}C-NMR δ ppm (DMSO-d_6): 46.869; 104.199; 105.490; 111.838; 112.097; 113.266; 114.178; 117.889; 118.201; 123.604; 126.248; 126.868; 131.498; 132.687; 152.796; 155.974; 158.291; 161.758. MS (ESI +) m/z: 392.2 (M + H, 100%). Anal. Calcd for $C_{20}H_{17}F_4N_3O$: C, 61.37; H, 4.37; N, 10.73. Found: C, 61.07; H, 4.44; N, 10.64.

4.1.6. (5-Methoxy-1H-indol-2-yl)(4-(4-(trifluoromethyl)phenyl)piperazin-1-yl)methanone (6)

Yield: 49%; mp.: 198-200 °C. ^{1}H-NMR (400 MHz, DMSO-d_6); δ ppm: 3.42 (t, 4H, piperazine-H), 3.75 (s, 3H, -OCH_3), 3.91 (bs, 4H, piperazine-H), 6.76 (d, 1H, J_m=2 Hz, H-3), 6.85 (dd, 1H, J_m=2.8 Hz, J_o=9.2 Hz, H-6), 7.07 (d, 2H, J_o=8 Hz, H-2′,6′), 7.08 (d, 1H, J_m=2), 7.32 (d, 1H, J_o=8.4 Hz, H-7), 7.53 (d, 2H, J_o=8.4 Hz, H-3′,5′), 11.47 (s, 1H, N-H). ^{13}C-NMR δ ppm (DMSO-d_6): 46.869; 55.220; 101.894; 104.005; 112.905; 114.147; 114.406; 117.843; 118.155; 123.604; 126.240; 127.101; 130.027; 131.209; 152.812; 153.749; 162.070. MS (ESI +) m/z: 404.4 (M + H, 100%). Anal. Calcd for $C_{21}H_{20}F_3N_3O_2$: C, 62.52; H, 4.99; N, 10.41. Found: C, 62.76; H, 5.34; N, 10.10.

4.1.7. (5-Fluoro-1H-indol-2-yl)(4-(4-fluorobenzyl)piperazin-1-yl)methanone (7)

Yield: 18%; mp.: 192-193 °C. ^{1}H-NMR (400 MHz, DMSO-d_6); δ ppm: 2.41 (t, 4H, piperazine-H), 3.49 (s, 2H, -CH_2-), 3.72 (bs, 4H, piperazine-H), 6.73 (d, 1H, J_m=1.6, H-3), 7.02 (t, 1H), 7.11-7.16 (m, 2H, H-3′,5′), 7.31-7.36 (m, 3H), 7.39 (dd, 1H, J_m=4.8 Hz, J_o=8.8 Hz), 11.63 (s, 1H, N-H). ^{13}C-NMR δ ppm (DMSO-d_6): 52.465; 60.820; 103.849; 105.375; 111.739; 113.144; 114.868; 126.813; 130.677; 131.645; 132.575; 133.899; 155.908; 158.222; 160.063; 161.531; 162.474. MS (ESI +) m/z: 356.5 (M + H, 100%). Anal. Calcd for $C_{20}H_{19}F_2N_3O$: C, 67.59; H, 5.38; N, 11.82. Found: C, 67.49; H, 5.37; N, 11.73.

4.1.8. (4-(4-Fluorobenzyl)piperazin-1-yl)(5-methoxy-1H-indol-2-yl)methanone (8)

Yield: 19%; mp.: 163-165 °C. ^{1}H-NMR (400 MHz, DMSO-d_6); δ ppm: 2.42 (bs, 4H, piperazine-H), 3.50 (s, 2H, -CH_2-), 3.74 (s, 7H, piperazine-H and -OCH_3), 6.67 (d, 1H, J_m=1.6 Hz, H-3), 6.83 (dd, 1H, J_m= 2.4 Hz, J_o=8.8 Hz, H-6), 7.04 (d, 1H, J_m=2.4 Hz, H-4), 7.15 (t, 2H, H-3′,5′), 7.30 (d, 1H, J_o=8.8 Hz, H-7), 7.36 (m, 2H, H-2′,6′), 11.41 (s, 1H, N-H). ^{13}C-NMR δ ppm (DMSO-d_6): 52.546; 55.213; 60.859; 101.879; 103.730; 112.836; 114.238; 114.825; 115.039; 127.063; 130.180; 130.751; 131.132; 153.711; 160.112; 161.887; 162.520. MS (ESI +) m/z: 368.5 (M + H, 100%). Anal. Calcd for $C_{21}H_{22}FN_3O_2$: C, 68.64; H, 6.03; N, 11.43. Found: C, 68.42; H, 6.12; N, 11.38.

4.1.9. (4-(4-Chlorophenyl)piperazin-1-yl)(5-methoxy-1H-indol-2-yl)methanone (9)

Yield: 35%; mp.: 191-192 °C. ^{1}H-NMR (400 MHz, DMSO-d_6); δ ppm: 3.21 (t, 4H, piperazine-H), 3.75 (s, 3H, -OCH_3), 3.88 (bs, 4H, piperazine-H), 6.75 (d, 1H, J_m=1.2 Hz, H-3), 6.85 (dd, 1H, J_m=2.4 Hz, J_o=8.8 Hz, H-6), 6.97 (d, 2H, J_o=9.2 Hz, H-2′,6′), 7.07 (d, 1H, J_m=2.4 Hz, H-4), 7.26 (d, 2H, J_o=9.2 Hz, H-3′,5′), 7.32 (d, 1H, J_o=8.4 Hz, H-7), 11.46 (s, 1H, N-H). ^{13}C-NMR δ ppm (DMSO-d_6): 48.732; 55.714; 102.386; 104.393; 113.376; 114.845; 117.621; 123.231; 127.572; 129.175; 130.560; 131.689; 149.982; 154.239; 162.504. MS (ESI +) m/z: 370.4 (M + H, 100%), 372.5 (M + 2, 33%). Anal. Calcd for $C_{20}H_{20}ClN_3O_2$: C, 64.95; H, 5.45; N, 11.36. Found: C, 65.11; H, 5.65; N, 11.38.

4.1.10. (5-Methoxy-1H-indol-2-yl)(4-(4-nitrophenyl)piperazin-1-yl)methanone (10)

Yield: 37%; mp.: 224-226 °C. ^{1}H-NMR (400 MHz, DMSO-d_6); δ ppm: 3.61 (s, 4H, piperazine-H), 3.75 (s, 3H, -OCH3), 3.92 (s, 4H, piperazine-H), 6.76 (s, 1H, H-3), 6.85 (d, 1H, J_o=8.4 Hz), 6.98 (d, 2H, J_o=8.8 Hz, H-2′,6′) 7.07 (s, 1H), 7.33 (d, 1H, J_o=9.2 Hz), 8.07 (d, 2H, J_o=8.8 Hz, H-3′,5′), 11.38 (s, 1H, N-H). ^{13}C-NMR δ ppm (DMSO-d_6): 45.787; 55.205; 101.970; 104.081; 112.227; 112.844; 114.406; 125.638; 127.094; 129.928; 131.201; 136.931; 153.726; 153.168; 162.093. MS (ESI -) m/z: 379.5 (M - H, 100%). Anal. Calcd for $C_{20}H_{20}N_4O_4 \cdot 2H_2O$: C, 57.68; H, 5.8; N, 13.45. Found: C, 57.68; H, 5.90; N, 13.49.

4.1.11. (5-Fluoro-1H-indol-2-yl)(4-(4-hydroxyphenyl)piperazin-1-yl)methanone (11)

Yield: 45%; mp.: 234-236 °C. ^{1}H-NMR (400 MHz, DMSO-d_6); δ ppm: 3.02 (t, 4H, piperazine-H), 3.86 (bs, 4H, piperazine-H), 6.67 (d, 2H, J_o=8.8 Hz, H-2′,6′), 6.80 (d, 1H, J_m=1.6 Hz, H-3), 6.83 (d, 2H, J_o=9.2 Hz, H-3′,5′), 7.05 (td, 1H, J_m=2.4 Hz, J_o=9 Hz), 7.36 (dd, 1H, J_m=2.4 Hz, J_o=9.6 Hz), 7.42 (dd, 1H, J_m=4.8 Hz, J_o=8.6 Hz), 8.89 (s, 1H, OH), 11.71 (s, 1H, N-H).^{13}C-NMR δ ppm (DMSO-d_6): 28.969; 30.363; 50.557; 103.989; 105.456; 111.845; 113.217; 115.496; 118.460; 126.857; 131.658; 132.649; 143.843; 151.417; 155.966; 158.283; 161.636. MS (ESI +) m/z: 340.1 (M + H, 100%). Anal. Calcd for $C_{19}H_{18}FN_3O_2 \cdot 0.5C_6H_{14} \cdot 0.3H_2O$: C, 68.12; H, 6.65; N, 10.83. Found: C, 68.15; H, 6.43; N, 10.49.

4.2. *In vitro* antioxidant activities

4.2.1. Superoxide radical scavenging activity (Cytochrome C Assay)

Superoxide radical scavenging activities of the synthesized compounds was determined according to a previously described method [39]. All experiments were carried out in triplicate and the results were given as

a percentage of the control. Vit-E was used as a positive control. Superoxide radical scavenging capacity was calculated using the formula given below:

$$\text{Superoxide radical scavenging activity (\%)} = [(A_{control} - A_{test}) / (A_{control} - A_{blank})] \times 100 \quad \text{(Eq. 1)}$$

$A_{control}$= the absorbance of the control excluding test compounds; A_{test}= is the absorbance of the test compound; A_{blank}= the absorbance of the blank excluding test compounds and the superoxide radical generating system.

4.2.2. DPPH free radical scavenging activity

The free radical scavenging activity of the compounds was determined using a previously described method with DPPH radicals [40]. Each experiment was carried out in triplicate and α -tocopherol was used as standard. The ability to scavenge DPPH radicals was calculated from the following equation:

$$\text{DPPH free radical scavenging activity (\%)} = [(A_{control} - A_{test}) / A_{control}] \times 100 \quad \text{(Eq. 2)}$$

A_{test}= the absorbance of DPPH radical and compounds; $A_{control}$= the absorbance recorded for methanolic solution of DPPH and DMSO solution excluding test compounds.

4.2.3. Lipid peroxidation

The effect of the synthesized compounds on rat liver homogenate induced with $FeCl_2$-ascorbic acid, and LP was measured by the method descibed previously [41]. α-Tocopherol was used as a standard. Each experiment was performed in triplicate. Lipid peroxidation inhibitory activity (%) was calculated from the folowing equation:

$$\text{LP inhibitory activity (\%)} = [(A_{control} - A_{test}) / (A_{control} - A_{blank})] \times 100 \quad \text{(Eq. 3)}$$

$A_{control}$= the absorbance of the control excluding test compounds; A_{test}= the absorbance of the test compounds; A_{blank}= the absorbance of the blank excluding test compounds and the free radical generating sytem (Fe^{+2} / ascorbate).

4.2.4. Anti-inflammatory activity

Fresh whole human blood was collected from healthy human volunteer who did not take any anti-inflammatory or steroidal drug for 2 weeks prior the experiment. The tubes were centrifuged at 3000 rpm for 10 min. The packed cells were washed with equal volume of isosaline (0.85%, pH 7.2). The volume of the blood was measured and reconstituted as 10% v/v suspension with isosaline. The membrane stabilizing activities of the compounds were evaluated using heat induced human erythrocyte hemolysis, designed by Anosike et al and Debnath et al with minor modifications. The reaction mixture consisted of equal volume of test sample and 10% red blood cells suspension. All the centrifuge tubes containing reaction mixture were incubated at 56 ^{o}C for 30 min. At the end of the incubation the tubes were cooled. The reaction mixture was centrifuged at 2500 rpm for 5 min. Then the absorbance of the supernatant was measured at 560 nm. The experiment was performed in triplicates for all the test samples. The results were expressed as the half maximal inhibitory concentration (IC_{50}) [42, 43]. **ASA** was used as a standard drug. The percentage hemolysis and protection was calculated according to the formula:

$$\text{Hemolysis\%} = (\text{Absorbance}_{\text{test sample}} / \text{Absorbance}_{control}) \times 100 \quad \text{(Eq. 4)}$$

$$\text{Protection\%} = 100 - [(\text{Absorbance}_{\text{test sample}} / \text{Absorbance}_{control}) \times 100] \quad \text{(Eq. 5)}$$

4.3. Molecular docking and molecular properties prediction

Molecular docking was carried out by using AutodockVina program. X-ray crystal structure of COX-2 (PDB ID: 3NT1) and the relative ligand were downloaded from Protein Data Bank (http://www.rcsb.org). AutoDock-Tools 1.5.6 (ADT) was used for preparing the pdbqt files. The protein was optimized by removing

water and co-crystallized ligand. Then polar hydrogens were added. Compounds were also energetically minimized. A grid box of 16×16×16 Å (x, y, and z) was created around the enzyme active pocket with the spacing of 1 Å and has the average coordinates of the crystallographic ligand in the pdb structure. Exhaustiveness was set to 10. Other vina docking parameters were set to default. The 3D compound-protein docking posses were analyzed manually using AutoDockTools.

Physicochemical features such as topological surface area, number of rotatable bonds and hydrogen bond acceptors & donors and Log P were calculated using Mol inspiration online tool [36].

4.4. Statistical analysis

The Statistical Package for the Social Sciences (SPSS) version 25.0 was used to perform the statistical analysis. All experiments were done in triplicate and the results were expressed as mean±SD. For the data, the analysis of variance was used to determine whether there are any significant differences between the means of the groups (ANOVA, Tukey's test). The differences between the groups were evaluated with Kruskal-Wallis test. $p < 0.05$ was considered statistically significant.

Acknowledgements: We thank Prof. Hakan Goker and Assoc. Prof. Mehmet Alp from Central Instrumentation Laboratory of Faculty of Pharmacy, Ankara University for NMR and elemental analysis.

Author contributions: Concept – T.G.A., Z.K.K.; Design – T.G.A., Z.K.K.; Supervision – T.G.A., Z.K.K.; Resources – T.G.A., Z.K.K.; Materials – T.G.A., Z.K.K.; Data Collection and/or Processing – A.B., C.A, Z.K.K, T.Ç., S.Y.S; Analysis and/or Interpretation – Z.K.K., C.A., T.Ç., S.Y.S; Literature Search – Z.K.K., C.A., A.B.; Writing – Z.K.K., C.A.; Critical Reviews – T.G.A., A.B., Z.K.K., C.A., S.Y.S., T.Ç.

Conflict of interest statement: The authors declare no conflict of interest.

Ethics committee approval: Lipid peroxidation protocols were approved by Gazi University Animal Experiments Local Ethics Committee, Ankara-Turkey (02.11.2016/G.Ü.ET-16.067). Anti-inflammatory activity protocol was approved by the ethics committees of the Faculty of Medicine of Ankara University, Ankara-Turkey (26.10.2015/16-695-15).

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