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RESEARCH ARTICLE

Phenolic Derivatives with Antioxidant and Anti-Inflammatory Activities: An in Silico, in vitro and in vivo Study

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A B S T R A C T

Polyphenols are widely distributed in nature having broad spectrum of biological activities. Most of the biological effects produced by polyphenols are largely attributed to their anti-inflammatory and antioxidant potential. The current research work describes the anti-inflammatory and antioxidant potential of newly synthesized phenolic derivatives (T2, T5, T6, T7 & T8) through *in silico*, *in vitro* and *in vivo* approach. The docking studies were performed against NADPH oxidase (PDB ID 3A1F-) and cyclooxygenase (COX-2 with PDB ID 5KIR Homosapien) using *in silico* techniques. Amongst phenolic derivatives (T2, T5, T6, T7 &T8) T2 and T6 exhibited maximum binding affinity with target protein NADPH oxidase (-6.2 and -6.3 Kcal/mol respectively). However, in case of COX-2 as target protein, all the five newly synthesized phenolic derivatives showed encouraging results. In particular, T2 and T8 with binding affinity values -7.9 Kcal/mol and -8.0 Kcal/mol respectively which are promising than Ibuprofen with binding affinity -6.6 Kcal/mol. Then *in vitro* antioxidant potential of phenolic derivatives was analyzed through total reducing power, total antioxidant capacity and DPPH (1, 1-diphenyl-2picryl-hydrazyl) assay. T2 and T6 showed antioxidant potential with IC50 values of 25.03µg/ml and 37.21 µg/ml respectively as compared to 15.14 µg/ml with respect to ascorbic acid. *In vivo* anti-inflammatory potential was determined through carrageenan induced paw edema which resulted in T2 with promising percentage edema inhibition (68.64±3.34) as compare to standard piroxicam (83.84±3.87). Amongst all phenolic derivatives T2 has promising results in *in silico*, *in vitro* and *in vivo* studies.

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INTRODUCTION

Reactive oxygen species (ROS) such as hydroxyl radical (OH•), superoxide anion radical and superoxide anion (O_2), in excess amount can contribute to wide range of disease including cardiovascular disorders and cancer (Goszcz*et al*., 2016). Numerous ailments including cancer, diabetes, vascular injury and heart diseases have direct pathological association with reactive oxygen species produced during inflammatory mechanisms. These free radicals may disrupt proteins, nucleic acid and other cell structures and may lead to tissue damage. Inflammation is a multifactorial process which is primarily associated with proteins denaturation and increased vascular permeability. Inflammatory mediators are released upon injury to the cells. These inflammatory mediators leads to activation of natural body's defense cells a process termed as chemotaxis. Chronic inflammation, results into a progressive movement of cells towards the site of inflammation which resulted into destruction of the tissues. So, the use of anti-oxidant and anti-inflammatory compounds may protect the cell from damage caused by oxidative stress (Somani & Bhattachar, 2014).

Polyphenolic compounds impart neuro protective as well as cardioprotective roles (Vauzour*et al*., 2010) Phenolic compounds inhibit pro-inflammatory molecules such as nitric oxide (NO) and tumor narcotic factor alpha showing their therapeutic worth in inflammatory diseases (Sanegar*et al*., 2015). Several phenolic compounds including oleuropein glycoside, vanillic acid, p-coumaric acid, syringic acid, homovanillic acid, caffeic acid, and tyrosol have inflammatory activity by inhibiting inflammatory cytokines and inflammatory eicosanoid PGE-2 (Miles*et al*., 2005). Our compounds are derivatives of methoxy phenols and several derivative of methoxy phenols like 2-tert-butyl-4-methoxyphenol (BHA), 2-methoxyphenols, 2,2'-dihydroxy5,5'-dimethylbiphenol and 2,2'-dihydroxy-5,5'- dimethoxy biphenol are already investigated for antioxidant and anti inflammatory activity (Kadoma*et al*., 2010).

Eugenol, ferulic acid and zingeron are also reported for their antioxidant and anti-inflammatory potential. Eugenol has ability to reduce CYP2E1 activity, lipid peroxidation indices, protein oxidation and inflammatory markers they also abolish the gene expression for Cyclooxygenase-2 (Yogalakshmi*et al*., 2010).

Our work reveals the antioxidant and anti-inflammatory potential of newly synthesized phenolic derivatives which may be useful against the ROS induced diseases such as cancer and cardiovascular diseases. The discovery of any new molecule is pricy and time taking process, but the integration of computational screening with the experimental data can change the discovery process of new drug molecules (Ekins*et al*., 2007).

MATERIALS AND METHODS

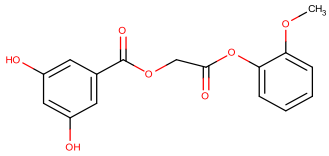
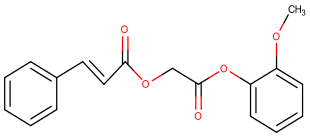
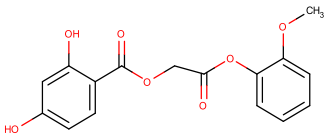
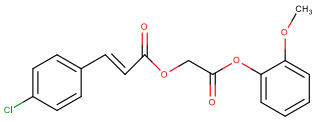
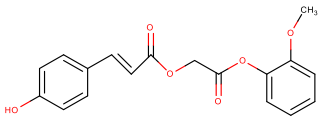
Chemistry of compounds: These are newly synthesized methoxy phenol derivatives having 2-methoxyphenoxy ring. The main core in all the newly synthesized derivatives is same the difference is the number and presence of –OH group at the benzene ring. These derivatives are then evaluated for their antioxidant and anti-inflammatory potential using *in silico*, *in vitro* and *in vivo* model. Their structure and IUPAC name are mentioned in Table 1.

Animals: Adult male BALB/c mice weighing 20-30g were housed under controlled environmental condition (23±1°C, 55±5% humidity and a 12-h light/dark cycle) were used. Animals were given free access to standard laboratory diet and to water.

In silico Assay

Target protein acquisition and preparation: The structures of the target proteins (NADPH oxidase, NOX-2 & cyclooxygenase-2, COX-2) was acquired from Protein Data Bank as PDB format (PDB ID 3a1f-Homosapien, PDB ID 5KIR Homosapien) (Berman *et al.*, 2000) (www.rcsb.org/pdb). These protein structures were validated through mol probity server. The accelrys discovery studio was used to remove water molecules and ligands. The UCSF chimera was used to minimize the energy of protein. The active binding sites of the proteins were viewed using PyMOL (Python molecular visualization software) (Reetz and Carballeira, 2007).

Table 1: Chemistry of phenolic derivatives

Phenolic derivative	Structure	IUPAC Name
T2		2-(2-methoxyphenoxy) -2-oxoethyl 3,5-dihydroxybenzoate
T5		2-(2-methoxyphenoxy) -2-oxoethyl (2*E*)-3-phenylprop-2-enoate
T6		2-(2-methoxyphenoxy) -2-oxoethyl 2,4-dihydroxybenzoate
T7		2-(2-methoxyphenoxy) -2-oxoethyl (2*E*)-3-(4-chlorophenyl) prop-2-enoate
T8		2-(2-methoxyphenoxy) -2-oxoethyl (2*E*)-3-(4-hydroxyphenyl) prop-2-enoate

Ligand preparation: Thenewly synthesized phenolic derivatives structures were drawn in Marvin sketch, cleaned into 3D structures and downloaded as PDB file (Ferrando*et al.*, 2012).

Docking: The virtual screening tool was used to predict *in silico* interaction of phenolic derivatives (ligand) with targets. The target proteins and ligands were then converted from protein data bank (pdb) to protein data bank quality treated (pdbqt) files by addition of hydrogen and removal of water molecules. The Chimera software was used to minimize the ligand and target interaction**.** These prepared files were then uploaded as ligand and target protein in PyRx, a python based auto dock vina. The autogrid dimensions were calculated for NADPH oxidase (NOX-2), cyclooxygenase (COX-2) and targets were geometrically optimized and docked (Aqeel *et al.*, 2018).

In vitro antioxidant activity

Total anti oxidant capacity: The total antioxidant capabilities of phenolic derivatives was measured by phosphomolybdenum assay as per described by Umamaheswari and Chatterjee (2008), with some modifications. The protocol started by adding 0.1 mL of the sample with that of 1 mL of the reagent solution. This reagent solution was prepared with 0.6 M H_2SO_4 and 28 mM Na_3PO_4 with 4 mM ammonium molybdate. Then the reaction mixture was treated with hot water (95°C) for 90 min. The reaction mixture was fully covered with silver foil to avoid direct light exposure. Then this reaction mixture was cooled and subjected to spectrophotometric analysis at 765 nm. The standard drug used was ascorbic acid.

Total reducing power: The methodology already described by Halliwell and Gutteridge (1981), was followed. 100 µL of phenolic derivative (T2, T5, T6, T7 & T8) was mixed with 500 µL of 2-deoxyribose (2.8 Mm) which was made by 50 mM phosphate buffer at pH 7.4, 100 µL of 200 mM H_2O_2, EDTA 0.1M and 200 µL ferric chloride (100mM). Then 100 µL of ascorbic acid (300 mM) was added and incubated at 37°C for 1 h. 1mL of 2.8% TCA and 1 mL of 1% w/v TBA, prepared in 50 mM NaOH were added to the reaction mixture. Then the final mixture heated for 15 min at water bath, cooled and subjected to spectrophotometer for evaluation at 532 nm. The scavenging activity was calculated by the following formula:

Scavenging activity (%) = (1- Absorbance of sample) / (Absorbance of control) X 100

DPPH assay: Later on, DPPH was performed following the methodology of Williams *et al*. (1995), to further confirm the presence of antioxidants. DPPH is based on the formation of yellowish diphenyl picrylhydrazine molecule when DPPH moiety is reduced by accepting a hydrogen or electron from a donor. Then 24 mg of DPPH in 100 ml methanol was prepared as stock solution and kept at 20°C. 100 mL of phenolic derivatives (T2, T5, T6, T7 & T8), (25-250 µg/ml) was mixed with DPPH (3mL) and optical density was checked at 517nm. Ascorbic acid was use as standard. The following equation was followed to determine the antioxidant capacity.

Scavenging effect (%) = (Absorbance of control – Absorbance of sample) / (Absorbance of control) X 100

***In vivo* anti-inflammatory activity**

Carrageenan-induced paw edema test: Paw edema was induced in Balb/c mice by intraplantar injection of carrageenan (0.1mL of 1% solution w/v in 0.9% saline) into sub-plantar area marked at left hind paw (100µl/paw) (Khalid*et al.,* 2018). These animals were divided into 8 different experimental groups (n=5) as vehicle control, negative control, positive control, treatment T2, treatment T5, treatment T6, treatment T7, treatment T8. Treatments were provided 45 min before carrageenan injection. Paw edema was evaluated at 0, 2, 4, and 6 h. The average difference of paw thickness (mm) of treated groups was compared to control groups (N.S. treated).

Statistical analysis: The values are presented as Mean ± SEM (n=4) with superscript (a-c) letters on the bars (P value <0.05) (LSD, Least significant difference).

RESULTS

In silico assay: In present study our newly synthesized phenolic derivatives were strongly entrapped in the active binding sites of NOX-2 and visualized through discovery studio as shown in Fig. 1. T2 attached with amino acids Ser91, Gln142, Glu135 having distance 3.09A^0, 2.24 A^0 and 2.38 A^0 respectively as shown in Fig. 2. Encouraging binding affinities were also found especially with T2 and T6 (-6.2 Kcal/mol and -6.3 Kcal/mol respectively) as shown in Table 2.

Table 2: Binding affinities of phenolic derivatives with NOX-2

Ligand	NOX-2	Binding affinity	rmsd/ub	rmsd/lb
T2		-6.2	0	0
T5		-4.9	0	0
T6		-6.3	0	0
T7		-6.1	0	0
T8		-5.2	0	0

Table 3: Binding affinities of phenolic derivatives with COX-2

Ligand	COX-2	Binding affinity	rmsd/ub	rmsd/lb
T2	-	-7.9	0	0
T5	-	-6.4	0	0
T6	-	-7.1	0	0
T7	-	-7.8	0	0
T8	-	-8.0	0	0
Ibuprofen	-	-6.6	0	0

Further all the phenolic derivatives were screened against COX-2 for anti-inflammatory potential. The derivatives showed promising results, especially T2 and T8 with binding energies-7.9 Kcal and -8.0 Kcal respectively which are significantly promising than Ibuprofen with binding energies -6.6 Kcal, as shown in Table 3. All the phenolic derivatives were tightly bound at active binding sites of COX-2 (Fig. 3). T2 also tightly bound to enzyme COX-2 and showed hydrogen bond interaction with amino acids Phe142 and Arg376 at distance of 2.46 A^0 and 2.36 A^0 respectively as shown in Fig. 4.

***In vitro* antioxidant activity**

Total Anti-oxidant Capacity (TAC): On the basis of phosphomolybdenum complex formation total antioxidant capacity of phenolic derivatives was calculated. TAC was shown by all the compounds in dose dependent manner as shown in Fig. 5. T5 showed the highest activity with level of significant of 0.001 at all the doses, followed by the T2, T6, T8 and T7. T2 also produce highly significant result at concentration of 31.25 µg/ml and 62.50 µg/ml with level of significance comparable to T5. T6 and T7 showed the comparable activity almost in all concentrations. Least significant result was also shown by T6 and T7 with level of significance of P<0.05 at concentration of 125 µg/ml and 250 µg/ml.

Total Reducing Power (TRP): In this test depending upon the reducing power of compound, the test solution changed to different colors. Some degree of reducing power was shown by almost all the tested compounds as shown in Fig. 6. Once again T5 (P<0.001) showed the highest reducing power (94.54%), than the other compounds (T2, 87.23%), at all the given concentration (31.25-250 µg/ml) in dose dependent manner.

DPPH assay: In this assay again T2 showed the most significant result and yielded the greatest DPPH scavenging activity followed by the T8, T5, T6 and T7 respectively, almost in all concentrations other than the 15.75 µg/ml. At this concentration T6 also produced the most significant result (42.65%) that was comparable to T2 (43.12%). At 125 µg/ml and 250µg/ml T6 (62.45%, 71.1%) showed the comparable result with T8 (63.24%, 67.77%) with same level of significant. Similarly, at concentration of 15.75 µg/ml, 31.25 µg/ml and 62.50 µg/ml DPPH scavenging activity was almost same for T7 (37.5%, 44.34%, 51.78%) and T8 (38.91%, 44.77%, 51.56%) (Fig. 7).

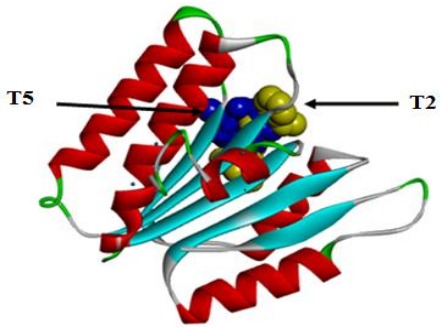


Fig. 1: T2 and T5 at the active binding sites of NOX-2.

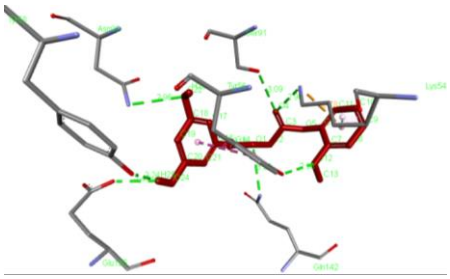


Fig. 2: The figure represents interaction of amino acids Ser, Gln, Glu with T2, visualized via Discovery Studio.

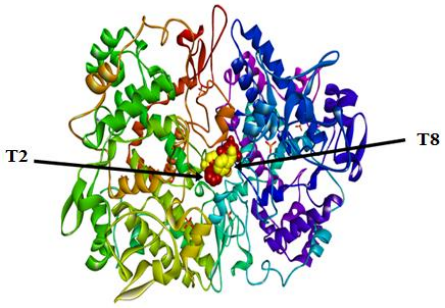


Fig. 3: T2 and T8 at the active binding sites of COX-2.

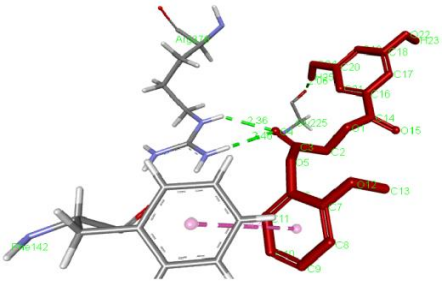


Fig. 4: The figure represents interaction of amino acids Phe&Arg with T2, visualized via Discovery Studio.

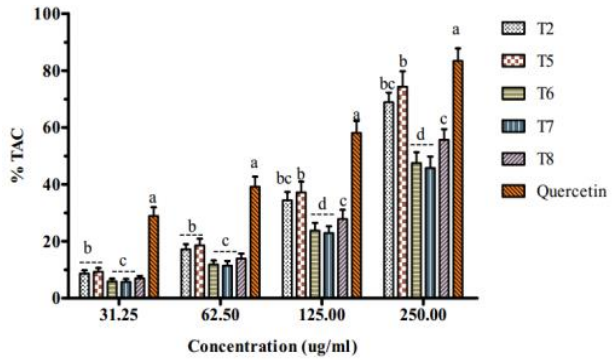


Fig. 5: The values represent Mean±SEM (n=3) the bars (P value <0.05).

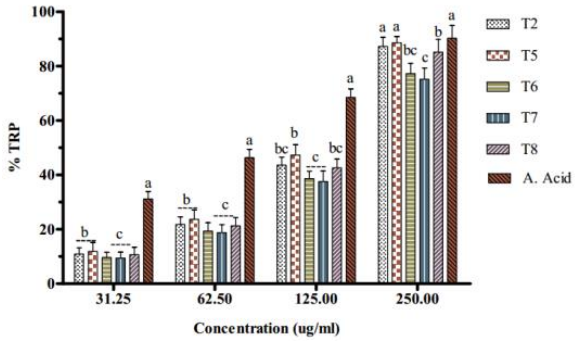


Fig. 6: The values represent Mean±SEM (n=3), superscripts (a-d) onsuperscripts (a-c) on the bars (P value <0.05).

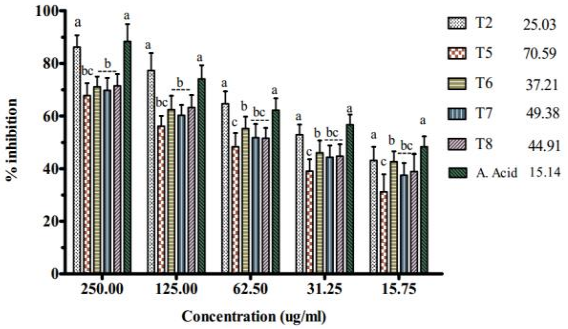


Fig. 7: The values represents Mean±SEM (n=3), superscript (a-c) letters on the bars are significantly (P<0.05) different from each other.

Table 4: Anti-inflammatory activity observed at different intervals of time

Groups	Edema volume (ml) Percent edema inhibition			
	0 h	2nd h	4th h	6th h
T2 (10mg/kg)	0.53±0.12	0.9±0.09	0.81±0.04	0.50±0.04
	0	27.75±3.54^{b}	52.30±3.02^{b}	68.64±3.34^{b}
T5 (10mg/kg)	0.56±0.07	1.03±0.12	0.78±0.08	0.52±0.05
	0	17.12±7.23^{d}	53.60±3.04^{b}	66.97±2.79^{b}
T6 (10mg/kg)	0.53±0.19	0.97±0.12	1.11±0.11	0.77±0.08
	0	21.90±3.96^{c}	34.16±6.64^{d}	51.56±5.32^{d}
T7 (10mg/kg)	0.53±0.13	1.01±0.06	1.19±0.06	0.88±0.12
	0	18.13±5.11^{cd}	29.23±5.47^{e}	43.71±6.07^{e}
T8 (10mg/kg)	0.51±0.14	0.91±0.08	0.98±0.11	0.67±0.04
	0	25.77±2.95^{bc}	41.85±9.01^{c}	57.22±3.27^{c}
Piroxicam (10mg/kg)	0.456±0.11	0.592±0.06	0.464±0.08	0.256±0.07
	0	52.04±2.81^{a}	72.45±5.37^{a}	83.84±3.87^{a}
Carrageenan control	0.54±0.13	1.236±0.12	1.692±0.16	1.574±0.06
Saline control	0.48±0.14	0.266±0.07	0.136±0.04	0.1±0.04

In vivo **anti-inflammatory activity:** All phenolic derivatives were screened for anti-inflammatory potential by using carrageenan-induced paw edema test. T2 and T8 also showed significant result after 2nd hour with percentage inhibition of 27.75 and 25.77% respectively. But after 4th and 6th hour results were not much significant for T8 then T2. T5 also showed comparable result with T2 after 4th and 6th hour but results were not statistically significant at 2nd hour. T7 exerted the least anti-inflammatory effect for 4th and 6th hour as compared to other group with level of significance of less the 0.05 (Table 4).

DISCUSSION

Interest for new anti-inflammatory and antioxidant agents have risen since past few decades as the pathophysiology of numerous age related diseases i.e.

diabetes, cancer, cardiovascular related diseases, Parkinson's disease and Alzheimer's have direct link with free radicals and ROS (Rahal *et al*., 2014). The chronic diseases associated with the production of free radicals, can be prevented by the inhibition of oxidative stress and ample interest in isolation and synthesis of new antioxidant and anti-inflammatory agents is emerging. This relationship between chronic diseases and inflammation has been reiterated by many authors (Hussain*et al*., 2016).

The present research work emphasizes on determining the antioxidant and anti-inflammatory potential of some newly synthesized methoxy phenol derivatives. The newly synthesized methoxy phenol derivatives were initially screened against NADPH oxidase enzyme of type 2 (NOX-2) using auto dock vina. Total antioxidant capacity (TAC), total reducing power (TRP) and DPPH assays were used for evaluation of antioxidant capacity through *in vitro* design of study. Additionally, the anti-inflammatory activity was performed by using carrageenan-induced paw edema test after *in silico* study against target COX-2. NOX family comprises of seven members NOX-1, NOX-2, NOX-3, NOX-4, NOX-5, DUOX-1 and DUOX-2 (Sorce*et al*., 2017). NOX enzymes can result into oxidative tissue damages in a number of diseases such as CNS disorders, diabetic complications and cardiovascular diseases (Fulton and Barman., 2016). The structure of NOX-2 and COX-2 protein was downloaded from protein data bank and active binding sites were identified as shown in Fig. 1 and Fig. 2 respectively. Our methoxy phenol derivatives showed encouraging binding affinities against NOX-2, T2 and T6 having binding affinities - 6.2 Kcal/mole and - 6.3 Kcal/mole as shown in Table 2. T2 and T8 also showed good binding affinities against target COX-2 (-7.9 Kcal/mole, -8.0 Kcal/mole) as compared to standard ibuprofen (-6.6 Kcal/mole) as shown in Table 3. In vitro anti oxidant study also showed encouraging results especially T2 showing IC_{50}value of 25.03 µg/ml as compare to standard ascorbic acid with IC_{50} value of 15.14 µg/ml (Fig. 7). T2 and T8 exhibited promising percentage edema inhibition (68.64±3.34, 66.97±2.97) in paw edema test as compared to standard piroxicam showing 83.84±3.87 percentage inhibition (Table 4).

Our compounds have structural resemblance with eugenol, which is a phenolic compound and main component of clove oil (Gulcin, 2011). Eugenol decreases the protein oxidation, lipid peroxidation and inflammatory markers in thioacetamide (TA)-induced hepatotoxic rats (Chang *et al*., 2010). Other than phenolic ring, benzoic acid ring is also present in our compounds which have also been investigated in many studies to possess antioxidant and anti-inflammatory activity. The presence of phenolic ring, benzoic acid ring and unsaturation in the side chains are making our compounds better antioxidant and anti-inflammatory candidate, producing comparable result to parent compound (Periera *et al*., 2009).

Conclusions: According to the data obtained methoxyphenol derivatives were found to contain effective antioxidant and anti-inflammatory potential. Henceforth, these newly synthesized phenolic derivatives can be considered as effective candidate for further development as potent antioxidant and anti-inflammatory agents.

Authors contribution: All authors contributed equally to the study design, experimental work, statistical analysis and writing of manuscript. The authors critically analyzed and agreed on the final version of manuscript.

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