

Research Paper

An *in silico* investigation on the inhibitory potential of the constituents of Pomegranate juice on antioxidant defense mechanism: Relevance to neurodegenerative diseases



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ABSTRACT

Elevation in the levels of reactive oxygen and nitrogen species (RONS), and downregulation of cellular antioxidants, have ubiquitously been reported from studies in animal models of neurodegenerative diseases, including Parkinson's disease (PD) and Alzheimer's disease (AD). Thus, plant-derived compounds are widely being investigated for their beneficial effects in these models. However, while studies have reported antioxidant potentials of several phytochemicals, a large number of studies have demonstrated different phytochemicals to be rather pro-oxidant and exaggerate oxidative stress (OS). One such study aimed to investigate possible ameliorative effect of Pomegranate juice (PJ) in rat model of toxin-induced parkinsonism revealed that PJ exacerbates OS, inflammation and promotes neurodegeneration. Thus, it remains to be investigated whether different constituents and metabolites of PJ are pro-oxidant or anti-oxidant. Using computational modeling, we investigated possible inhibitory potential of different constituents of PJ and their metabolites viz. delphinidin-3-glucoside, dimethylelagic acid-glucuronide, ellagic acid, ellagitannin, gallic acid, gallotannin 23, pelargonidin, punicalagin, urolithin A, urolithin A-glucuronide and urolithin B, on anti-oxidant defense system of the brain. The results indicate that the constituents of PJ have the potential to inhibit five key enzymes of the neuronal antioxidant defense system, viz. catalase, superoxide dismutase, glutathione peroxidase 4, glutathione reductase and glutathione-S-transferase. Thus, it is surmised that the constituents of PJ may contribute to OS and neurodegeneration by way of affecting antioxidant defense mechanism. This may particularly be more pronounced in neurodegenerative diseases, since neurons are known to be more vulnerable to OS. Thus, the present findings caution the use of PJ in patients prone to OS, especially those suffering from neurodegenerative diseases, and warrant further experimental studies to unveil the effects of individual components and metabolites of PJ on antioxidant defense system of brain.

1. Introduction

Oxidative stress (OS) is caused by an increase in the cellular levels of reactive oxygen and nitrogen species (RONS). In normal physiological conditions, RONS play important role in cellular defense. However, excessive production of RONS damage macromolecules, cause cell death (Lee et al., 2012), and has been identified to be a key factor behind neuronal demise in diseases including Alzheimer's disease (AD) (Kempainen et al., 2015; Melief et al., 2015) and Parkinson's disease (PD) (Dexter and Jenner, 2013; Beal, 2005; Lipski et al., 2011). The major sources of the RONS are the NADPH oxidase, xanthine oxidase and mitochondria, and thus mitochondrial dysfunctions have been reported to cause OS and neurodegeneration in animal models of PD and

AD (Beal, 2005; Jiang et al., 2016; Kim et al., 2015; Subramaniam and Chesselet, 2013; Lipski et al., 2011; Leszek et al., 2016; Borah et al., 2013; Bhattacharjee et al., 2016; Schapira, 2009; Dauer and Przedborski, 2003).

The cellular homeostatic redox state is maintained by a balance between RONS and antioxidants. The antioxidants are both enzymatic as well as non-enzymatic components. The enzymatic components include superoxide dismutase (SOD), catalase, glutathione-S-transferase (GST) and glutathione peroxidase (GPx) (see Kim et al., 2015 for a review). Reduced glutathione (GSH) is the most potent non-enzymatic cellular antioxidant, and is also used by GST and GPx to neutralize a wide range of oxidants. SOD catalyses the conversion of superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2), and catalase degrades

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H₂O₂ into water. GST catalyses conjugation of GSH with a large array of oxidants (Ålin et al., 1985; Hubatsch et al., 1998; Van der Oost et al., 2003; Awasthi et al., 2004; Sharma et al., 2004; Hayes et al., 2005) and is known to reduce the levels of RONS (Siems et al., 1997; Siems and Grune, 2003; Balogh and Atkins, 2003; Bastos et al., 2013). GPx 4 is the most widely expressed GPx isoform in neurons and glia (Zhang et al., 2010; Wang et al., 2013), and is implicated in neurodegenerative diseases (Bellinger et al., 2011). GPx 4 detoxifies a range of products of lipid oxidation, including nitrotyrosine, nitrotocopherol, malondialdehyde, acrolein and 4-hydroxynonenal (Williamson et al., 2002; Hall et al., 2004; Perluigi et al., 2009; Pizzimenti et al., 2013; Pitts et al., 2014). During OS, GSH is oxidized to glutathione disulphide (GSSG), and GSH is recycled by the enzyme glutathione reductase (GR). Thus, GR is vital in maintaining GSH pool in brain (Schafer and Buettner, 2001; Couto et al., 2016).

Since OS has been found to be ubiquitous in animal models of neurodegenerative diseases, as well as in several other diseases, use of antioxidant therapy has gained tremendous focus in PD and AD research. In this context, plant-derived compounds have largely been investigated for their ameliorative effects. Several studies have reported protective role of phytochemicals, including curcumin, tea polyphenols, caffeine, resveratrol, etc. in ameliorating OS in animal models of toxin-induced parkinsonism (Fujisawa et al., 2004; Guo et al., 2007; Yu et al., 2010; Kachroo et al., 2010; Deb et al., 2019; see Seidl et al., 2014 for a review). In primary human neurons, Pomegranate juice (PJ) extract was found to regulate redox metabolism and thereby confer neuroprotection (Braidly et al., 2013). The neuroprotective effects of PJ have been studied in animal model of AD as well, which reported inhibition of aggregation of amyloid and amelioration of behavioral abnormalities (Hartman et al., 2006).

On the contrary, there are reports that phytochemicals can potentiate OS and neurodegeneration in animal models. It is suggested that polyphenols may cause H₂O₂ and O₂^{·-}-mediated OS (Vittal et al., 2004; Yen et al., 2004; Krych and Gebicka, 2013). Tapias et al (2014) have reported that administration of PJ extract in rodent model of toxin-induced PD results in exaggeration of OS and elevation in the nitrotyrosine level, accompanied by activation of caspase-3 and NF-κB and degeneration of dopaminergic neurons. High doses of PJ were reported to cause OS in rodent model of sepsis (Tavasoli et al., 2019). Thus, there is lack of consensus from studies involving animal models on the use of PJ in ameliorating OS. We hypothesize that although the phytochemicals present in the PJ may scavenge free radicals, different

compounds may interact and interfere with the activities of the enzymes of the antioxidant defense mechanism, and thereby contribute to OS. We have validated the hypothesis using computational modeling approach.

2. Methodology

2.1. The receptors

For the present study, five enzymes of the cellular anti-oxidant mechanism, viz. catalase, SOD, GPx, GR and GST, were selected to estimate the inhibitory potential of the constituents and metabolites of PJ on these. Three-dimensional structures of human erythrocyte catalase 3-amino-1,2,4-triazole complex (PDB id: 1DGH), human SOD 1 complexed with isoproterenol (PDB id: 5YTU), human GPx 4 in complex with GXpep-1 (PDB id: 5H5Q), human GR in complex with a xanthine inhibitor (PDB id: 1XAN) and human GST complexed with sulfasalazine (PDB id: 13GS) were downloaded from Protein Databank (www.rcsb.org/pdb) in .pdb format. All the five structures are crystal structures determined using X-ray diffraction at resolutions of 2 Å, 1.9 Å, 1.1 Å, 2 Å and 1.9 Å respectively. Selection of the structures was based on resolution, source organism and availability of bound ligand for reference of active site, and the residues of the active site.

2.2. The ligands

Twelve compounds, comprising constituents of the PJ and the metabolites of the constituents, were selected for the modeling analysis, based on literature review (Table 1). The known inhibitors of the receptors (or enzymes) were selected based on available literature, viz. hydroxylamine (for catalase), isoproterenol (for SOD), tiopronin (for GPx 4), 3,6-dihydroxy-xanthene-9-propionic acid (for GR) and sulfasalazine (for GST). β-carotene and limonene, both of which are plant-derived antioxidant molecules but without any hydrogen bond forming functional groups, were included in the modeling analysis as negative controls for hydrogen bonding. Three-dimensional structures of all the ligands were downloaded from NCBI PubChem compounds database (www.pubchem.ncbi.nlm.nih.gov) in .sdf format. Details of the ligands with their properties are given in Table 1. Since three-dimensional conformers of Punicalagin and Ellagitannin were not available at the database, two-dimensional conformers, bearing IDs 44584733 and 10033935, were downloaded from the database. The structures were

Table 1

Details of the ligands used in the study. HBD: Number of Hydrogen bond donor; HBA: Number of Hydrogen bond acceptor; PJ: Pomegranate juice; SOD: superoxide dismutase; GST: glutathione-S-transferase; GR: glutathione reductase and GPx: glutathione peroxidase 4.

Compound Name	PubChem Compound ID	Molecular Weight (in g/mol)	HBD	HBA	Rotatable Bond Count	Topological Polar Surface Area	Formal Charge	Type of ligand
Hydroxylamine	787	33.03	2	2	0	46.2	0	Catalase inhibitor
Isoproterenol	3779	211.261	4	4	4	73	0	SOD inhibitor
Sulfasalazine	5339	398.393	3	9	6	150	0	GST inhibitor
3,6-Dihydroxy-xanthene-9-propionic acid	449159	286.283	3	5	3	87	0	GR inhibitor
Tiopronin	5483	163.191	3	4	3	67.4	0	GPx inhibitor
Delphinidin-3-glucoside	443650	465.387	9	11	4	202	1	PJ constituent
Dimethylellagic acid-Glucuronide	101419926	506.372	5	14	5	208	0	PJ metabolite
Ellagic acid	5281855	302.194	4	8	0	134	0	PJ constituent
Ellagitannin	10033935	992.713	13	27	5	447	0	PJ constituent
Gallic acid	370	170.12	4	5	1	98	0	PJ constituent
Gallotannin 23	12795683	636.471	11	18	10	311	0	PJ constituent
Pelargonidin	440832	271.248	4	4	1	81.9	1	PJ constituent
Urolithin A	5488186	228.203	2	4	0	66.8	0	PJ metabolite
Urolithin A-Glucuronide	102579638	404.327	5	10	3	163	0	PJ metabolite
Urolithin B	5380406	212.204	1	3	0	46.5	0	PJ metabolite
Punicalagin	44584733	1084.722	17	30	0	511	0	PJ constituent
β-carotene	5280489	536.888	0	0	10	0	0	Negative control
Limonene	22311	136.238	0	0	1	0	0	Negative control

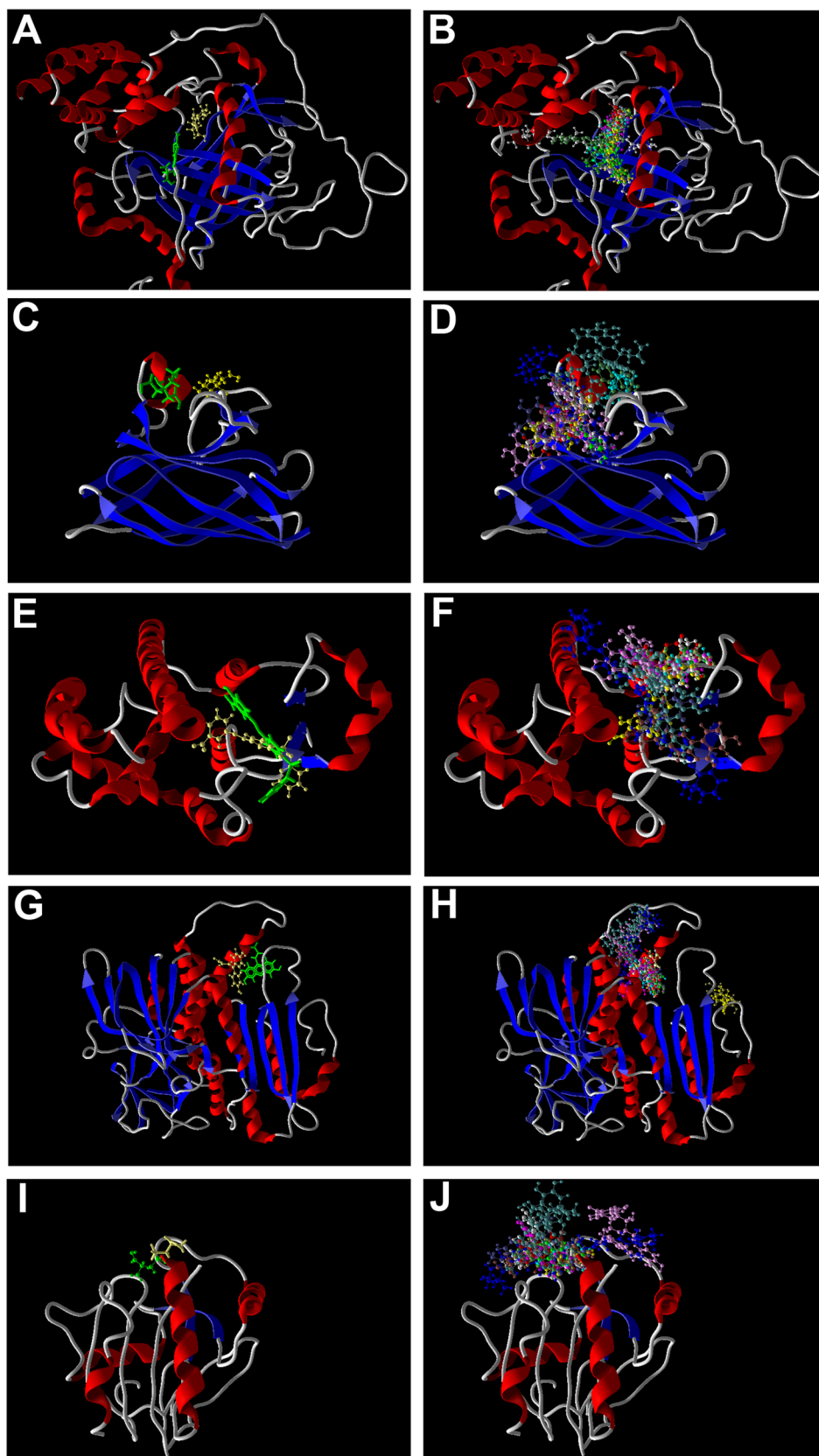


Fig. 1. Docking poses of the ligands with the active sites of the receptors: A-B: catalase; C-D: superoxide dismutase; E-F: glutathione-S-transferase; G-H: glutathione reductase; I-J: glutathione peroxidase 4. The yellow coloured ligands in the left column (A, C, E, G, I) are the docked poses of the co-crystallized ligands, while the other ligands in these images are the actually co-crystallized poses available with the PDB structures. The docking of the co-crystallized ligands at the same active site where they were co-crystallized shows accuracy of the computational modeling study. The right column (B, D, F, H, J) shows all the phytochemicals and metabolites of PJ docked at the active site of the receptors. The poses were obtained following docking using Molegro Virtual Docker 2.1 software. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
The docking scores (MolDoc and Hydrogen bond scores) of the different ligands with the respective receptors. The known inhibitors of the receptors are Hydroxylamine, Isoproterenol, Sulfasalazine, 3,6-Dihydroxy-xanthene-9-propionic acid and Tiopronin for the receptors catalase, superoxide dismutase, glutathione-S-transferase, glutathione reductase and glutathione peroxidase 4 respectively. The scores were obtained by docking using Molegro Virtual Docker 2.1 software.

Compounds	Catalase		Superoxide dismutase		Glutathione-S- transferase		Glutathione reductase		Glutathione peroxidase 4	
	Docking Score	H Bond Score	MolDoc Score	H Bond Score	MolDoc Score	H Bond Score	MolDoc Score	H Bond score	MolDoc Score	H Bond score
Known Inhibitor	-22.949	-6.056	-69.054	-7.980	-114.198	-5.876	-94.853	-4.737	-65.171	-5.719
Delphinidin-3-glucoside	-141.073	-15.041	-115.212	-13.777	-118.619	-13.456	-128.380	-15.536	-97.415	-7.544
Dimethylglucic acid-Glucuronide	-140.234	-10.344	-92.760	-9.421	-110.797	-13.019	-116.177	-7.797	-92.366	-9.594
Ellagic acid	-99.358	-4.632	-72.897	-9.183	-82.291	-9.681	-86.320	-8.069	-86.774	-5.898
Ellagittannin	-91.135	-10.805	-130.598	-11.246	-156.405	-14.829	-126.484	-11.366	-112.198	-12.042
Gallic acid	-70.151	-11.704	-59.516	-10.778	-79.654	-8.743	-61.668	-6.668	-77.073	-7.640
Gallotannin 23	-169.200	-15.922	-108.091	-14.041	-142.044	-16.153	-141.310	-13.384	-92.438	-8.990
Pelargonidin	-105.240	-7.276	-94.019	-7.482	-87.683	-4.956	-88.445	-6.540	-83.474	-6.383
Urolithin A	-93.901	-3.512	-71.402	-5.018	-81.794	-6.482	-81.425	-5.283	-87.000	-4.006
Urolithin A-Glucuronide	-130.455	-7.134	-92.816	-12.170	-100.761	-10.315	-111.680	-9.719	-101.522	-8.301
Urolithin B	-88.582	-2.357	-66.799	-4.839	-75.284	-4.986	-73.427	-3.333	-81.402	-2.623
β -carotene	-112.462	0.000	-146.695	0.000	-142.916	0.000	-168.051	0.000	-126.223	0.000
Limonene	-68.688	0.000	-55.325	0.000	-78.290	0.000	-57.263	0.000	-68.550	0.000

converted to three-dimensional conformers using ChemOffice software package, followed by energy minimization, saved in .mol format, and then used for the modeling analysis.

2.3. Molecular docking

Molecular docking is a powerful computational modeling tool in evaluating binding of a ligand (phytochemicals or others) to the active site of an enzyme or receptor. The free energy of binding of a ligand is taken as reference for comparison of binding of other unknown ligands, and possible inhibition of the enzyme. The amount of energy liberated upon docking of a ligand with a receptor is a measure of stability of the complex, and indicates how strongly a ligand may inhibit an enzyme (Paul and Choudhury, 2010; Mazumder et al., 2013, 2014, 2015, 2018a, 2018b; Mazumder and Borah, 2014, 2015; Mazumder and Choudhury, 2019). In the present study, molecular docking was performed between the active sites of the enzymes and the selected phytochemicals of the PJ, using the respective known inhibitors as the reference ligands, following Mazumder et al. (2013, 2014, 2015, 2018a, 2018b). The docking was performed using Molegro Virtual Docker 2.1 package, which uses MolDock / PLP scoring function (Thomsen and Christensen, 2006). Briefly, individual structures of the enzymes were loaded into the software, followed by addition of the PJ phytochemicals and the respective reference ligand (known inhibitor) into the workspace. All water molecules available with the receptors were removed from the workspace. The ligands were docked taking the co-crystallized ligand as reference, and with a docking radius of 20 Å around the reference ligand. Maximum number of iterations were set to 1500 and maximum number of runs was set to 10. The best docking score (MolDoc and Hydrogen bond score), in terms of free energy of binding (expressed as negative values), were considered for further analysis.

2.4. Statistical analysis

To analyze which ligand property is vital in inhibiting the respective receptor, correlation analysis was performed using Microsoft Office Excel 2007. The correlation of the docking (MolDoc) scores obtained for the different receptors were studied against different properties of the ligands (viz. molecular weight, number of hydrogen bond donor and hydrogen bond acceptor groups present, topological polar surface area, number of rotatable bonds and formal charge). The ligand(s) which did not dock at the active sites of the receptors were discarded from the statistical analysis.

3. Results

3.1. Interaction of the ligands with the receptors

The computational modeling revealed that all the compounds, except punicalagin, could effectively bind with the active ligand binding sites of the respective receptors. Against GR, the best pose of all ligands except β -carotene and urolithin A, in terms of MolDoc score, bind to the same active site of the receptor. In case of GPx 4, all ligands except gallotannin 23, bind to the same site of the receptor. In case of all other receptors, all the ligands bind to the same active site as that of the co-crystallized ligand (Fig. 1). In the docking simulation, the co-crystallized ligands of the respective receptors were included. The docking results revealed that the co-crystallized ligand dock to the same site where they were co-crystallized, which demonstrates accuracy of the present modeling study (Fig. 1). Further, binding of the ligands to the same site as that of co-crystallized ligand and the known inhibitor, indicates similar mode of inhibition of the receptors by all the compounds.

Table 3

Pearson's correlation co-efficient of MolDoc scores of each receptor with different properties of the ligands. HBD: Number of hydrogen bond donor groups; HBA: Number of hydrogen bond acceptor groups; NC: not correlated.

Receptor	Molecular weight	HBD	HBA	Rotatable bond count	Topological polar surface area	Formal charge
Catalase	−0.575	−0.504	NC	−0.656	NC	NC
Superoxide dismutase	−0.823	NC	NC	−0.772	NC	NC
Glutathione-S-transferase	−0.929	−0.640	−0.671	−0.876	−0.673	NC
Glutathione reductase	−0.758	NC	NC	−0.902	NC	NC
Glutathione peroxidase 4	−0.762	NC	NC	−0.647	NC	NC

3.2. Inhibition of activities of the enzymes

When a ligand binds to the active catalytic site of a receptor, it physically competes for the active site. Further, more the free energy of binding, i.e., docking score, more is the stability of the ligand-receptor complex. Thus, the ligand may block the receptor's activity (Paul and Choudhury, 2010; Mazumder et al., 2013, 2014, 2015, 2018a, 2018b; Mazumder and Borah, 2014, 2015; Mazumder and Choudhury, 2019). The present computational modeling study demonstrated that all the phytochemicals and / or metabolites of PJ, except punicalagin, could effectively interact with the active sites of the respective receptors (Fig. 1). Among the constituents and / or metabolites of PJ, gallocatechin 23 showed highest MolDoc scores against catalase and GR, while ellagitannin showed highest scores against SOD, GST and GPx 4. Further, gallocatechin 23 showed highest hydrogen bond scores against catalase, SOD and GST, while against GR and GPx 4, delphinidine-3-glucoside and ellagitannin showed highest hydrogen bonding score respectively (Table 2). It may be noted that, the ligands limonene and β -carotene showed zero hydrogen bond scores against all the receptors (Table 2), which was expected since these two compounds lack any hydrogen bond donor or acceptor group (Table 1).

Compared to the known inhibitors of the respective receptors, catalase, SOD, GST, GR and GPx 4, the MolDoc scores of the phytochemical / metabolite showing highest docking score were found to be 7.37 – fold, 1.89 – fold, 1.37 – fold, 1.49 – fold and 1.72 – fold respectively (Table 2).

3.3. Correlation of the docking scores with properties of the ligands

Statistical analysis revealed that molecular weight of the ligands is mildly negatively correlated to the MolDoc score of catalase, while for all other receptors the molecular weight was found to be strongly negatively correlated with MolDoc scores. Likewise, the number of rotatable bonds in the ligands was found to be negatively correlated to the MolDoc scores, for all the receptors. Number of hydrogen bond donor groups present in the ligands was found to be negatively correlated to the MolDoc score for the receptors catalase and GST, while number of hydrogen bond acceptor groups and topological polar surface areas were found to be negatively correlated to the MolDoc score for GST (Table 3). Formal charge of the ligands was found to be not correlated to the docking scores. It may be noted that since the docking scores are negative values, the Pearson's correlation coefficient for all the receptors against all the ligand properties were found to be negatively correlated.

4. Discussion

The present study was undertaken to enumerate the potential of different phytochemicals of PJ and their metabolites in affecting cellular anti-oxidant defense system, using computational modeling analysis. The results indicate that the different compounds, viz. delphinidine-3-glucoside, dimethylellagic acid-glucuronide, ellagic acid, ellagitannin, gallic acid, gallocatechin 23, pelargonidin, urolithin A, urolithin A-glucuronide and urolithin B, may potentially inhibit catalase, SOD, GST, GR and GPx (Table 2), by interfering with their active

catalytic sites (Fig. 1). However, the PJ constituent punicalagin showed positive docking scores against all the receptors, thereby indicating that it may not inhibit any of these. This may be because of the fact that this component of PJ is too large (with highest molecular weight of 1084.722 g/mol among all the compounds studied) to be accommodated at the active sites of the receptors. However, the metabolites of punicalagin, i.e. urolithin A and urolithin B, showed potentials in inhibiting all the enzymes (Table 2).

Statistical analysis revealed that molecular weight and number of rotatable bonds in the ligands are negatively correlated to the docking score for each receptor (Table 3). Further, the number of hydrogen bond donors in the ligands was found to be negatively correlated to the docking scores of catalase and GST, while number of hydrogen bond acceptor and topological polar surface area were found to be negatively correlated in case of GST (Table 3). Thus, molecular weight and number of rotatable bonds of the ligands are the principal properties which determine the inhibitory potential of the ligands. It may be noted that the higher docking scores are the more negative scores, and signifies better inhibition of the receptors (Mazumder et al., 2013, 2014, 2015, 2018a, 2018b), and since the docking scores are negative values, all the correlation coefficients are negative signifying negative correlation.

Inhibition of catalase and SOD may elevate the cellular levels of H_2O_2 and $O_2^{\cdot -}$, thereby leading to production of the RONS, like hydroxyl radical ($\cdot OH$) (Riederer et al., 1989; Double and Halliday, 2006). Thus, the findings suggest that these compounds may lead to H_2O_2 and $O_2^{\cdot -}$ - mediated OS, which is inline with studies with teapolyphenols (Vittal et al., 2004; Yen et al., 2004; Raza and John, 2005; Babich et al., 2007, 2011; Bhat et al., 2007). Inhibition of GPx and GST by the constituents of PJ and their metabolites may elevate the levels of RONS, including nitrotyrosine, which may explain the basis for the increase in the level of this species demonstrated by Tapias et al. (2014). Further, since GR is needed to maintain GSH pool in the neurons, inhibition of this critical enzyme may deplete the cellular GSH pool and thereby elevate the levels of RONS.

Thus, the present findings on the inhibitory potentials of PJ phytochemicals and their metabolites on the studied enzymes may be a useful explanation for the elevation in the nitrotyrosine level, exaggeration of OS and dopaminergic neurodegeneration in rat model of toxin-induced PD, as reported by Tapias et al. (2014). Several studies have reported rather pro-oxidant nature of phytochemicals, mainly polyphenolic compounds, including epigallocatechin-3-gallate, gallic acid, protocatechuic acid, syringic acid, vanillic acid, ellagic acid, coumaric acid, chlorogenic acid, ferulic acid, myricetin, quercetin, rutin, kaempferol, (+)-catechin, (−)-epicatechin, delphinidin, malvidin and caffeic acid, which includes OS, depletion of GSH and inhibition of anti-oxidant enzymes (Raza and John, 2005; Bhat et al., 2007; Babich et al., 2007, 2011). These phytochemicals have similar structures and functional groups as those of the different constituents of PJ and their metabolites. Thus, the present findings are in line with these reports that the constituents of PJ and their metabolites may inhibit antioxidant enzymes and contribute to depletion of GSH level, and thereby OS.

In view of the present findings, it is argued that although phytochemicals, like those of PJ, have antioxidant potency, they may inhibit cellular antioxidant defense mechanism as well. In other words, they

may act both as pro-oxidants as well as antioxidants. As such, their use as adjunct for cellular defense against OS is a major concern, mainly in neurodegenerative diseases since neurons are more vulnerable to OS. Thus, it is suggested that dose- and time- dependent *in vitro* and *in vivo* studies be carried out using individual constituents of PJ and their metabolites before they can be used as therapeutics or adjuncts against OS in neurodegenerative diseases, including PD and AD. Further, pharmacokinetic and pharmacodynamic studies are warranted for each of these phytochemical and metabolite.

Conflict of interest

We declare no potential conflict of interest in publishing the article.

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