Density functional theory-based quantum rationalization of flavones from *Oroxylum indicum*, their correlation with redox effect, molecular interaction studies and osmotic hemolysis

Nusrat Masood[1,#], Akhilesh Kumar Yadav[1,#], Naresh Kumar[2], Madan Mohan Gupta[1] and Suaib Luqman[1,*]

[1]CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow 226 015, India
[2]Central Institute of Plastic Engineering and Technology, Lucknow 226 008, India

Four flavones (chrysin, baicalein, oroxylin A and hispidulin) characterized from ethanolic root extract of *Oroxylum indicum* (a traditional dietary nutraceutical supplement), were compared with both experimental (radical scavenging action and osmotic fragility test on human erythrocytes) and theoretical (Density functional theory (DFT) (B3LYP/6-31G*) calculations and *in silico* docking with haemoglobin and albumin) for their redox properties. Raman spectra were examined specifically between 2900 and 3700 cm^{-1} and the corresponding peaks were identified for hydroxyl group stretching vibrations. Baicalein and hispidulin had the highest and lowest binding energy respectively for oxyhaemoglobin (oxyHb) and vice versa for deoxyhaemoglobin (deoxyHb), which was one of the major findings revealed in their superposed docked structures where the position of baicalein was not changed unlike hispidulin. On the whole, baicalein is the preeminent flavone as it revealed maximum activity in various antioxidant assays, protection against osmotic fragility and binding energy with oxyHb which can be reasoned out by its least HOMO–LUMO energy gap.

Keywords: Flavones, HOMO–LUMO, *Oroxylum indium*, osmotic fragility, Raman spectra.

OROXYLUM INDICUM vent. (Bignoniaceae), generally known as Syonakh, is consumed as a common part of the diet (fruits and flowers) in northeastern Asia and is utilized in Ayurvedic preparation 'Dashmool' and other formulations[1]. The plant is known to possess various pharmacological attributes like antiarthritic, antibacterial, antifungal, anti-inflammatory, antimutagenic, analgesic, diuretic and gastroprotective properties. Chemically, it includes many flavonoids, pterocarpanoids, phenylethanoids, cyclohexylethanoids and sterols[2–5]. Flavonoids are studied extensively as therapeutic functional food metabolite-based drugs for oxidative stress-mediated diseases and disorders. They are said to modulate and normalize biological homeostasis because of their polyphenolic nature. Structurally, they all possess a basic C_6–C_3–C_6 phenyl-benzopyran backbone. One of the prominent subgroups within the flavonoid group is flavones which bear a C_2–C_3 double bond and a C_4–oxo function[6]. The flavones selected for this study are Baicalein, Oroxylin A, Chrysin and Hispidulin, isolated and characterized from the root of *O. indicum* by our group[2] and others[7,8]. The basic ring structure with attached hydroxy groups, characteristic of chrysin, is also present in other three flavones (baicalein, oroxylin A and hispidulin, (Supplementary Figure 1).

Chrysin (5,7-dihydroxy-flavone), with myorelaxant and anticonvulsant properties, is a ligand for benzodiazepine receptors[9]. Chrysin is found to be effective as a cancer chemopreventive agent against benzo(a)pyrene-induced lung cancer in mice[10]. It also exhibits anti-inflammatory and antioxidant properties and is used as a dietary supplement[11] as well as in the anti-ageing clinical therapeutic application[12].

Baicalein (5,6,7-trihydroxy-flavone), in human lung carcinoma A549 cells, induces caspase-dependent apoptotic death via redox-mediated AMPK (5′ AMP-activated protein kinase) activation[13]. It also possesses cardioprotective, anticancer and anti-inflammatory properties as well as used in the treatment of symptoms like fever, copious perspiration and insomnia[14]. It protects membrane damage against deleterious free radicals mostly by acting as chain-breaking antioxidant. Nrf2 signalling-mediated apoptosis by baicalein was observed in C6 glial cells against oxidative stress due to hydrogen peroxide[15].

Oroxylin A (5,7-dihydroxy-6-methoxy-flavone) is an O-methylated flavone. It was first isolated by Naylor and

#Equally contributed.
*For correspondence. (e-mail: s.luqman@cimap.res.in)

Chaplin (1890) from the root bark of *O. indicum*. It is a dopamine reuptake inhibitor and a negative allosteric modulator of the benzodiazepine (BZD) site of $GABA_A$ receptors[16,17]. It improves memory consolidation through brain-derived neurotrophic factor (BDNF) levels in the hippocampus of mice[18]. Oroxylin A is also effective as an anti-inflammatory, antitumour, antibacterial and for cognitive enhancement[19].

Hispidulin (4′,5,7-dihydroxy-6-methoxy-flavone) is also an O-methylated flavone reported to be one of the ligands of benzodiazepine BZD site of $GABA_A$. It is also an anticancer, antihypnotic and antiepileptic agent. Density functional theory (DFT) study revealed the antioxidant capacity of hispidulin comparable to quercetin, indicating that the radical scavenging nature of hispidulin has the convincing competency to fight against reactive oxidizing species and also it does not violate the Lipinski's rule of five[20].

To the best of our knowledge, no report for radical scavenging, quantum analyses, molecular interaction studies and osmotic haemolysis of the erythrocytes of the selected flavones (differ only in the hydroxy and methoxy groups) from the root of *O. indicum* has been recorded. However, antioxidant activity of different extracts of *O. indicum* has been reported[21]. Therefore, we have studied the quantum rationalization of flavones using DFT-based approach and correlated it with the redox effect, molecular interaction studies and osmotic haemolysis of the erythrocytes.

Materials and methods

Chemicals

Aluminium chloride, L-ascorbic acid sodium salt, 2,2′-diphenyl-1-picrylhydrazyl, dimethyl sulphoxide, folin-ciocalteau reagent, 2,4,6-tri-pyridyl-triazine, trichloroacetic acid were obtained from Sigma-Aldrich, India. Ferric chloride, ferrous sulphate, naphthyl ethylenediamine dichloride, potassium acetate, potassium ferrocyanide, sulphanilamide, sodium carbonate, disodium phosphate salt, monosodium diphosphate salt, tris-base, meta-phosphoric acid, sodium nitroprusside, sodium and potassium phosphate buffer were obtained from Himedia Laboratories Pvt Ltd, India. All the reagents, chemicals and solvents used in the assays were of analytical grade.

Isolation and characterization

The isolation and characterization of chrysin, baicalein, oroxylin A and hispidulin from the roots of *O. indicum* has been reported earlier[2].

Biochemical assays

Ethanolic extract and the characterized flavones (chrysin, baicalein, oroxylin A and hispidulin) were subjected to antioxidant activity evaluation using different assays: (reducing potential (RP), ferric ion reducing antioxidant power (FRAP), total phenolic content (TPC), total antioxidant capacity (TAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), nitric oxide (NO) and total flavonoid content (TFC)[22]). A stock solution of extract (100 mg/ml) and four flavones (10 mg/ml) was prepared in dimethyl sulphoxide (DMSO), diluted in phosphate buffer saline (PBS) as required. Concentration-dependent studies were carried out (1, 10, 25 and 50 µg/ml) in replicates and osmotic fragility test[23] was performed at 10 and 100 µg/ml.

Quantum chemical studies

DFT calculations were performed with Gaussian 03 software with GaussView as the graphical interface at the B3LYP (Becke 3-parameter hybrid exchange functional and Lee–Yang–Parr correlation functional) level of theory and employing the 6-31G* basis set[24–26]. The 3D structures of baicalein (CID_5281605), chrysin (CID_5281607), hispidulin (CID_5281628) and oroxylin A (CID_5320315) were retrieved from PubChem database at NCBI web server (http://pubchem.ncbi.nlm.nih.gov) in the sdf format and converted to pdb format by OpenBabel 2.3.1 software[27]. The structures were optimized and later each optimized geometry was used to run the frequency calculation, Raman vibrational intensities and molecular orbital eigen value calculations. However, for a final view of HOMO (highest occupied molecular orbital), LUMO (lowest unoccupied molecular orbital) and ESP (electrostatic potential), Avagadro software[28] was used for their pictorial generation.

Molecular interaction studies

To comprehend the interactions of flavones with haemoglobin and albumin, docking studies were done. X-ray crystallographic 3D structures of oxyhaemoglobin (PDB ID: 2DN1, 1.25 Å resolution crystal structure of human haemoglobin in the oxy form), deoxyhaemoglobin (PDB ID: 2DN2, 1.25 Å resolution crystal structure of human haemoglobin in the deoxy form) and albumin (PDB ID: 4K2C, HSA ligand-free) were retrieved from Brookhaven Protein Data Bank (http://www.pdb.org). Docking was executed using AutoDock 1.5.4 software[29] as the version uses Lamarckian Genetic Algorithm and empirical free energy scoring function. AutoDock 1.5.4 uses a semi-empirical free energy force field to evaluate conformations in terms of inhibition constant (Ki) and binding energy (BE) during docking simulations. The docked proteins were visualized and superposition of docked oxyHb and deoxyHb proteins was done through UCSF Chimera, v. 1.6 (ref. 30) in order to understand the relative orientation and configuration of flavones. Also to understand the association between H-bonding and hydrophobic interactions,

Ligplot 1.4.5 (http://www.ebi.ac.uk/thrntosrv/software/LIGPLOT/) was used to engender schematic diagrams of protein–ligand interactions[31].

The AutoDock 1.5.4 program starts with flavone as ligand molecule and finds favourable docking in a protein-binding site using simulating annealing. Genetic algorithms, polar, polar hydrogens and Kollman charges were added to the protein. Grid centre was centered on the ligand binding site with 60 × 60 × 60 grid parameters and the grid spacing of 0.375 Å was calculated. All other parameters were followed as reported earlier[32].

Statistical analysis

Concentration-dependent experiments were done and an average of the values was reported with standard deviation obtained from three independent experiments in replicates. Correlation among antioxidant parameters was done by Pearson coefficient. The value of Pearson coefficient ranged from –1 to +1 showing significant negative or positive correlation between different biochemical assays.

Results and discussion

Antioxidant assays

The order of reducing power was baicalein > oroxylin A > hispidulin > chrysin (Figure 1 *a*). Baicalein having 3 hydroxy groups showed the highest reducing power compared to others. Although hispidulin too has 3 hydroxy groups, it also has a methoxy group. Both hydroxy and methoxy groups are activating functional groups when attached to a benzene ring but hydroxy group is a more influencing group. Additionally, the reducing power appears to be related to the degree of hydroxylation and extent of conjugation in polyphenols[33]. The order of reactivity towards FRAP assay expressed as µM of ferrous sulphate equivalence was, oroxylin A > baicalein > hispidulin > chrysin (Figure 1 *b*). The assay system detects compound with redox potentials of <0.7 V which is based on the reduction of [2,4,6-tri(2-pyridyl)-s-triazine (TPTZ)–Fe^{3+} (redox potential of Fe^{3+}–TPTZ: 0.7 V] to intense violet-coloured TPTZ-Fe^{2+} complex by an antioxidant measured at 593 nm. FRAP can be predicted for the ability of the sample to maintain redox status in the biological system but not for molecules that act by radical quenching or H-transfer[34]. Chrysin was the least reactive flavone in both assays involving the conversion of iron in the assay. The order of activity of selected flavones in total phenolic content (expressed in terms of µg gallic acid equivalence) was baicalein > oroxylin A > chrysin > hispidulin (Figure 1 *c*). Baicalein was having the highest phenolic content. However, hispidulin with least effect too had hydroxy groups but at different rings and also a methoxy group. The TAC was only shown by baicalein and the extract (Figure 1 *d*). Other flavones did not reveal TAC. The assay system measures both fat soluble and water soluble antioxidant capacity of flavones due to the formation of green phospho-molybdenum complex by reduction of Mo(VI) to Mo(V). Apart from TAC, baicalein showed better antioxidant property than other flavones even in other assays also. However, intriguing aspect in TAC was that the rest of the flavones did not show any activity in this assay. The order of DPPH radical scavenging was hispidulin > oroxylin A > baicalein > chrysin (Figure 1 *e*). The result showed that DPPH scavenging assay was guided by the number of both hydroxy groups and methoxy groups. Hispidulin contains three hydroxy groups and one methoxy group while oroxylin A contains two hydroxy groups and one methoxy group. However, baicalein and chrysin contain only three and two hydroxy groups respectively.

The order of NO radical scavenging assay was baicalein > chrysin > oroxylin A > hispidulin (Figure 1 *f*). This assay system showed that baicalein and chrysin which have only hydroxy groups as functional groups were more effective NO scavengers compared to oroxylin A and hispidulin which also have methoxy group along with hydroxy group as functional groups. The reaction of NO with phenolic groups may help to attenuate NO concentration which is not possible with the methoxy group. Therefore, this finding supports the hypothesis that O-methylation of hydroxy group results in decreased NO scavenging activity. The total flavonoid content expressed as quercetin equivalent was estimated in the extract and was found to exhibit activity in a concentration-dependent manner (Figure 1 *g*). Correlation among the antioxidant parameters was done by Pearson coefficient and the value ranged from –1 to +1 showing significant negative or positive correlation between different biochemical assays (Supplementary Table 1).

Osmotic fragility test

The extent of haemolysis of human erythrocytes was also measured by performing osmotic fragility test (Figure 1 *h*) at two selected concentrations (10 and 100 µg/ml). The increasing order of fragility tested at higher concentration was hispidulin < baicalein < oroxylin A < chrysin. However, the protective effect of baicalein was more pronounced at 10 µg/ml contrary to that of hispidulin revealing protection at 100 µg/ml. Both baicalein and hispidulin have three hydroxy groups while chrysin and oroxylin A have two. Similarly, hispidulin contains one additional methoxy group which may have a positive influence compared to the other three flavones. Hydroxy group is a more activating functional group than methoxy group when attached to the aromatic ring. The mean erythrocyte fragility values (MEF_{25}, MEF_{50} and

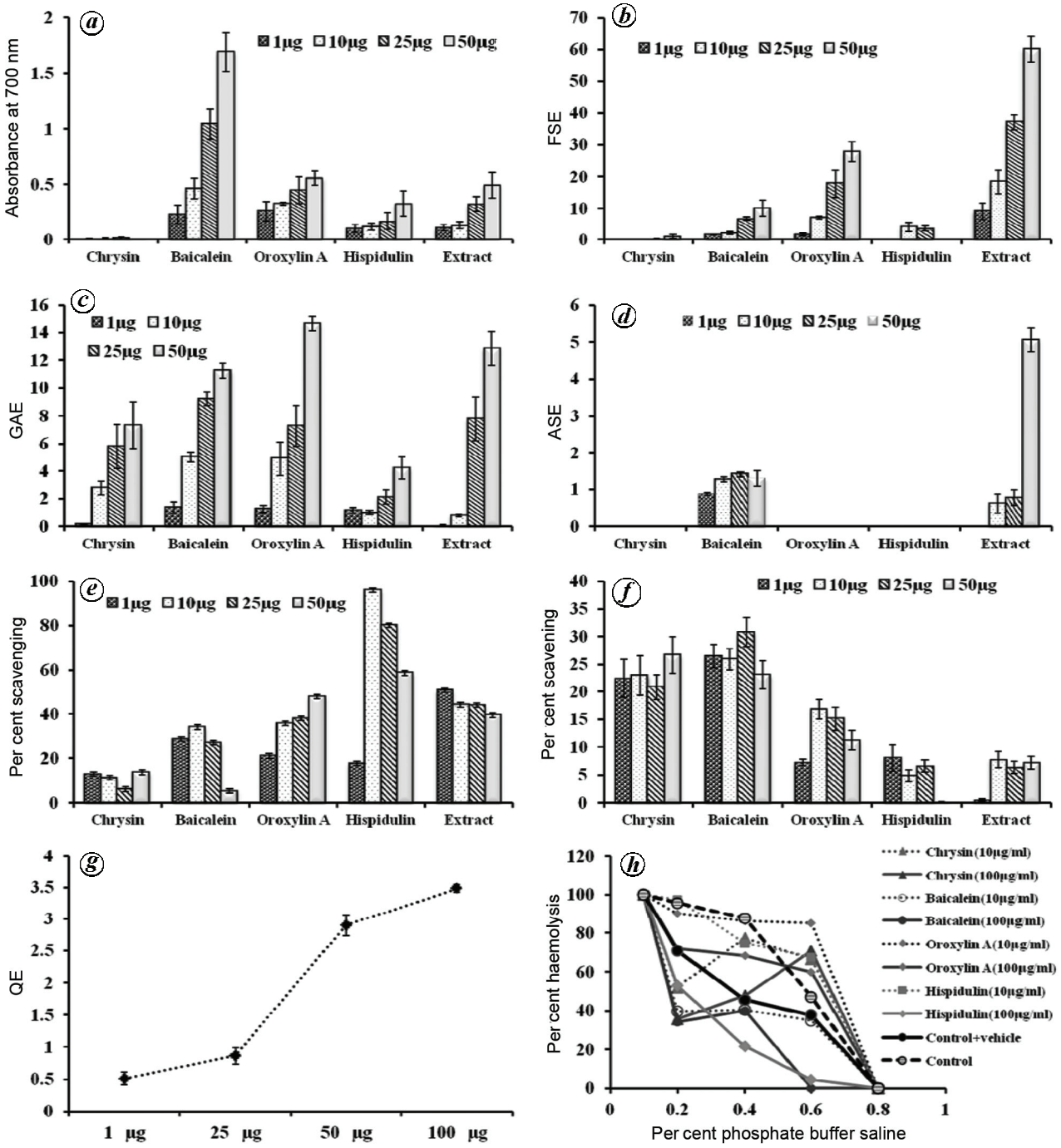

Figure 1. Concentration-dependent efficacy of selected flavones: ***a***, reducing power (expressed in terms of absorbance at 700 nm); ***b***, Ferric reducing antioxidant power (expressed as µM of ferrous sulphate equivalence, FSE); ***c***, Total phenolic content (expressed in terms of µg gallic acid equivalence, GAE); ***d***, Total antioxidant capacity (expressed in terms of µg ascorbic acid equivalence, ASE); ***e***, DPPH radical scavenging assay; ***f***, Nitric oxide scavenging assay; ***g***, Total flavonoid content of ethanolic extract (expressed in terms of µg quercetin equivalence, QE) and ***h***, Erythrocyte fragility profile of flavones. Values are mean ± SD of three independent experiments in replicates at each concentration.

MEF_{75}) are presented in Table 1. Erythrocytes being enucleated and biconcave in shape provide a well-built surface area to endure stress and force. Their colloidal character, elasticity and viscosity provide properties essential for numerous cellular functions. Exposure of erythrocytes to stress activates iNOS, an enzyme that exports nitric oxide which bestows the regulation of vascular tone[35]. Under stress, erythrocytes are lysed and the extent or degree of haemolysis can be measured through fragility profile that arises when erythrocytes are subjected to osmotic stress by putting them in a hypotonic solution[36]. Animal studies have revealed that the vulnerability of

dietary deficiencies increases erythrocyte fragility and addendum with antioxidants decreases osmotic haemolysis and oxidative damage of erythrocytes[37]. Flavonoids exert beneficial effects under oxidative stress by donating electrons for ferricyanide reduction supporting the protective effects of flavonols and their glycosides against 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH)-mediated free radical-induced oxidative haemolysis of erythrocytes[38]. Quercetin, cirsimaritin, rutin, luteolin, chrysoeriol-4′-O-glucoside, 3,5,7-trihydroxy 4′-methoxyflavone 7-rutinoside and β-naphtho flavone have been found to perk up erythrocyte osmotic fragility, suggesting the presence of a hydroxy group essential for the rheological effects of these flavonoids[39]. On the contrary, apigenin and diosmin treatment opposed the rise in erythrocyte rigidity and resulted in a dose-dependent decrease of the membrane cholesterol : phospholipid ratio[40,41]. Overall, baicalein was found to be the most protective in fragility test.

Quantum chemical descriptors

DFT is a computational method for calculating molecular properties based on the electron density of a molecule. B3LYP, run with a 6-31G* or a better basis set is usually preferred for most systems especially organic molecules. Various quantum chemical descriptors such as HOMO energy, LUMO energy, electron affinity (A), ionization potential (I), electronegativity (χ), quantum chemical hardness index (η), electrophilicity (ω), electrofugality (ΔE_e) and nucleofugality (ΔE_n) derived from quantum chemical calculations are presented in Table 2. These descriptors are used to understand the properties of molecules and their relationship with various biological phenomena, and are thus exploited in medicinal chemistry and QSAR predictions due to the versatility and reliability of calculations[42]. The frontier molecular orbital theory resolves the way molecule interacts with other groups resulting in the formation of a transition state, as the electrons from HOMO participate freely in the reaction with LUMO which is a good electrophilic site. The energy of HOMO (electron donor) and LUMO (electron acceptor) is directly related to ionization potential and electron affinity respectively. The difference in the energy gap of HOMO–LUMO represents an important molecular polarization index where a decrease leads to easier polarization of the molecule. The low energy gap is responsible for electron-transfer properties and electron transport in the molecule[43]. HOMO and LUMO electron densities determine electron donor and acceptor sites respectively. According to Koopmans' theorem, ionization potential is negative of HOMO energy, while electron affinity is negative of LUMO energy. Electron affinity refers to the capacity of a ligand to allow precisely one electron from a donor. Hispidulin has the highest HOMO as well as LUMO energy among the studied flavones, and thus have the least ionization potential and electron affinity. The frontier orbital theory helps in defining the reactivity of the molecules. Higher HOMO energy has more tendency to donate electrons and lower LUMO energy has more propensity to accept electrons. Baicalein has the least HOMO–LUMO energy gap which is necessary for reactivity while chrysin has the highest HOMO–LUMO energy gap. The electronic chemical potential refers to the escaping tendency of electrons from equilibrium and is identified as the negative of electronegativity, which is shown as $-\mu = -(I + A)/2 = -(\varepsilon\text{HOMO} + \varepsilon\text{LUMO})/2 = -\chi$.

Chrysin has the least chemical potential while hispidulin has the highest chemical potential which may be due to the hydroxy functional group attached to ring B which is absent in other three flavones. The greater the electronic chemical potential, the less stable or more reactive the molecule will be (Supplementary Figure 2). Electrophilicity index measures the global electrophilic power (capacity to accept electrons) of the molecule[44] and also evaluates energy stabilization of the system after an additional electronic charge is taken from the environment, i.e. lesser electrophilicity, more stability of the molecule[45]. Thus it is an index of reactivity or toxicity, e.g. toxicity of polychlorinated biphenyls and benzidine[46,47]. The equation of electrophilicity index is $\omega = \mu^2/2\eta = -\chi^2/2\eta$ and is can be expressed in terms of HOMO and LUMO as $\mu = -(\varepsilon\text{HOMO} + \varepsilon\text{LUMO})/2$ and $\eta = \varepsilon\text{LUMO} - \varepsilon\text{HOMO}$; where εLUMO and εHOMO represent the energies of the lowest unoccupied and highest occupied molecular orbital respectively. Baicalein has the highest while hispidulin has the lowest electrophilicity index. Chemical hardness measures the resistance to the change in electron density. The equation is $\eta = (I - A) = \varepsilon\text{LUMO} - \varepsilon\text{HOMO}$. Chemical hardness is associated with the stability and reactivity of a chemical system. The larger the HOMO–LUMO energy gap, the harder and more stable and less reactive the molecule and vice versa. In this study, chrysin and baicalein respectively have the highest and lowest HOMO–LUMO energy gap among the four flavones. Electrofugality ($\Delta E_e = I + \omega = (\mu - \eta)^2/2\eta$) and nucleofugality ($\Delta E_n = -A + \omega = (\mu + \eta)^2/2\eta$) are two recent reactivity indices related to electrophilicity and

Table 1. Mean erythrocyte fragility values of flavones from *O. indicum*

Flavones	Concentration (μg/ml)	MEF_{25}	MEF_{50}	MEF_{75}
Chrysin	10	0.775	0.702	0.485
	100	0.781	0.715	0.563
Baicalein	10	0.708	0.407	0.208
	100	0.52	0.324	0.164
Oroxylin A	10	0.788	0.73	0.672
	100	0.762	0.68	0.191
Hispidulin	10	0.778	0.708	0.444
	100	0.418	0.224	0.154
Control		0.745	0.635	0.520

Table 2. Quantum chemical descriptors of four flavones by density functional theory calculation at the B3LYP/6-31G* level

Properties	Chrysin	Baicalein	Oroxylin A	Hispidulin
HOMO (eV)	–6.215	–6.005	–5.999	–5.956
LUMO (eV)	–2.167	–2.196	–2.159	–2.06
HOMO–LUMO energy gap	4.048	3.809	3.84	3.896
Ionization potential (I)	6.215	6.005	5.999	5.956
Electron affinity (A)	2.167	2.196	2.159	2.06
Chemical potential (μ)	–4.191	–4.1005	–4.079	–4.008
Electronegativity (χ)	4.191	4.1005	4.079	4.008
Chemical hardness (η)	4.048	3.809	3.84	3.896
Electrophilicity index (ω)	2.169	2.207	2.1664	2.061
Electrofugality (ΔE_e)	8.384	8.212	8.1654	8.017
Nucleofugality (ΔE_n)	0.0023	0.011	0.0074	0.0016

Table 3. Residues surrounding the flavones within 4 Å of docked albumin–flavone complex and residues forming H-bond with the flavones

	Residues within 4 Å vicinity	H-Bond (electrostatic interactions)	Hydrophobic residues
Chrysin	Y 150, K 199, R 218, L 219, R 222, L 238, H 242, L 260, A 261, I 264, S 287, I 290 and A 291	Y150 : 3.164 Å; K199 : 2.689 Å; H242 : 2.641 Å	L 219, L 238, L 260, I 264, I 290 and A 291
Baicalein	Y 150, R 257, S 287, A 291, K 199, W 214, A 215, R 218, L 238, L 219, R 222, L 238, R 257, L 238, R 257, S 287, I 290 and A 291	Y150: 2.727Å, 1.82Å; S287: 3.196Å R257: 2.936Å	A 291, W 214, A 215, L 238, L 219, L 238, L 238, I 290 and A 291
Hispidulin	Y 199, W 214, A 215, R 218, L 219, R 222, L 238, H 242, A 261, I 264, S 287, I 290 and A 291	K199 : 2.695 Å ,3.554 Å; H242 : 2.644 Å, S 287 : 2.822 Å, 1.874Å	W 214, A 215, L 219, L 238, A 261, I 264, I 290 and A 291
Oroxylin A	Y 150, R 257, S 287, A 291, K 199, W 214, A 215, R 218, L 238, L 219, R 222, L 238, R 257, L 238, R 257, S 287, I 290 A 291, I 264, A 261, L 260 and H 242	Y150 : 3.127 Å; K199 : 2.739 Å H242 : 2.547 Å	A 291, W 214, A 215, L 238, L 219, L 238, L 238, I 290 A 291, I 264, A 261 and L 260

nucleophilicity, which measure the electron releasing and accepting abilities respectively. It is assumed that the electrofugality of a molecule is evaluated by its group nucleophilicity and therefore related to the electron releasing ability of the substituent attached to the electrofuge moiety while nucleophiles display a high group electrophilicity of the leaving group to depart with the bonding electron pair. These indices are important with respect to functional groups. Thus, it is evident that baicalein has the highest electrophilicity and nucleofugality index. In other words, electrofugality and nucleofugality are essentially group properties while electrophilicity and nucleophilicity refer to the whole molecule[48]. However, hispidulin has the least electrophilicity index, electrofugality as well as nucleofugality (Supplementary Tables 2 and 3 and Supplementary Figure 3).

Raman spectra

A comparison of Raman spectra of the studied flavones revealed various peaks, but we only focused on those peaks which corresponded to the hydroxyl stretching that was mainly responsible for the free radical scavenging potential. The Raman spectra of flavonoids were studied in which different flavonoid groups were considered and a simplification was drawn[49], but a specific study on hydroxy group of flavones was not done. Raman spectra of various flavones were individually studied and matched with the experimental data of some previous studies like 3-hydroxyflavone (3-HF) and 5-hydroxyflavone (5-HF) and quercetin (3,5,7,3′,4′ penta hydroxy flavone)[50]; study on the effect of pH on the chemical modification of quercetin[49]; study on 7-hydroxyflavone and 3′,4′-dihydroxyflavone by DFT calculations using B3LYP functional and 6-31+G* basis set[51]; study on chrysin, apigenin and luteolin, by DFT calculations using B3LYP functional and 6-31+G* basis set[7] and oroxylin by DFT at B3LYP/63-11G(d,p)[52].

We obtained the Raman spectra of chrysin, baicalein, oroxylin A and hispidulin which differed at the structure level only on the number of hydroxy and methoxy substituents on the benzene ring (Figure 2). The specific Raman spectra from 2900 to 3700 cm^{-1} enabled us to get intense individual peak of hydroxyl group stretching obtained from DFT calculations which were performed with Gaussian 03 at B3LYP level of theory and employing the 6-31G* basis set. The vibrational Raman spectrum is simple compared to infrared (IR) spectra and it

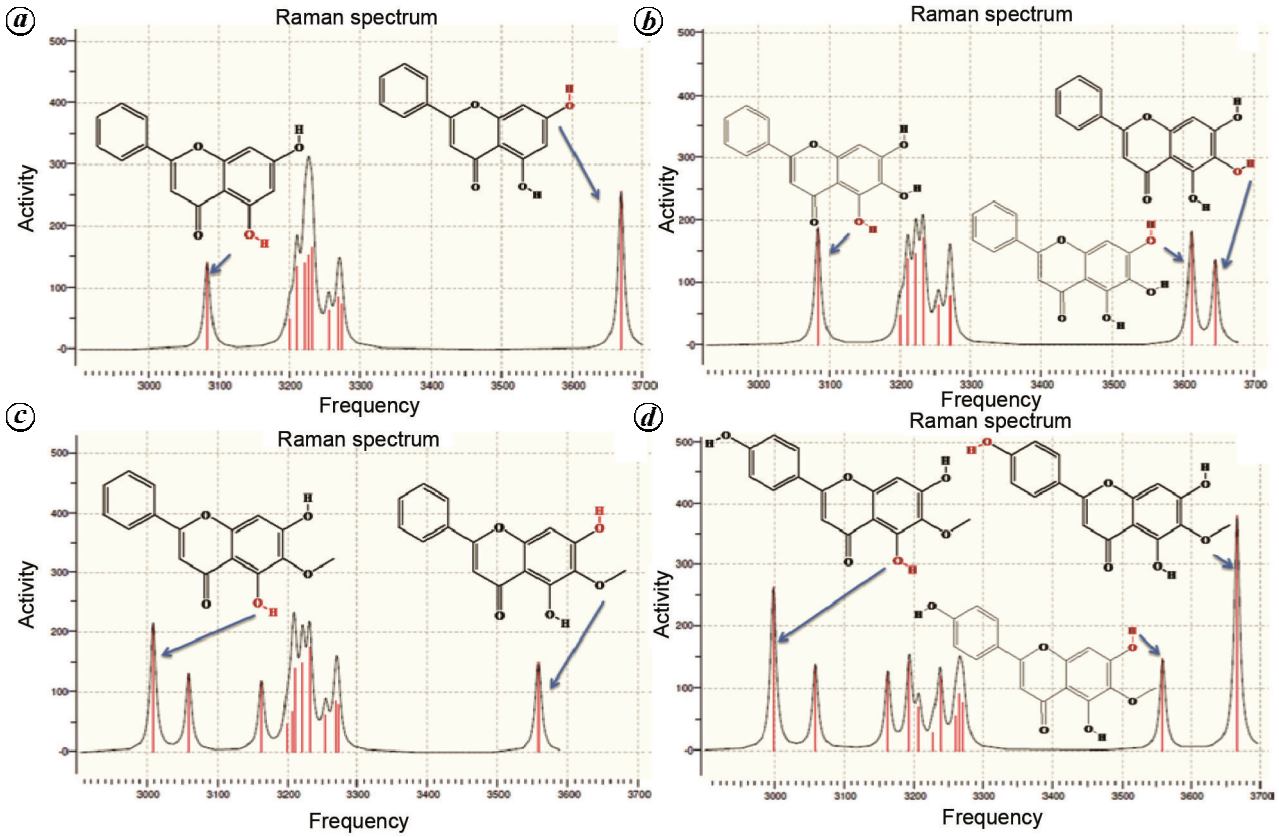

Figure 2. Specified plot of Raman spectra of the selected flavones calculated by DFT calculations using B3LYP/6-31G+* basis set done by Gaussian 03 software and viewed by the help of Gauss View 3.0. Only specified position from 2900 to 3700 cm^{-1} Raman frequency is being depicted at the *x*-axis while the *y*-axis corresponds to Raman activity in terms of intensity. Compared to IR spectra, Raman spectra were simpler and in this plot we have focused only those sharp bands which corresponded to the vibrational stretching of hydroxyl bond as depicted by the structure of flavones and the hydroxy group coloured in red: (***a***) Raman spectra of chrysin which have two hydroxy groups at ring-A correspondingly represented by two sharp peaks; (***b***) Raman spectra of baicalein which have three hydroxy groups in the same ring, i.e. ring-A represented by three sharp peaks; (***c***) Raman spectra of Oroxylin-A which have two hydroxy groups represented by two sharp peaks; (***d***) Raman spectra of hispidulin which have three hydroxy groups (two in ring-A and one in ring-B) represented by three sharp peaks.

reflects the molecular structure with specific signature functional group identification and therefore analyses the vibrational modes of molecules and functional groups, allowing bond characterization (Supplementary Table 4). In the case of flavonoids, vibrational spectroscopy was used to study hydroxyl and carbonyl groups[53]. IR spectra are more complex than Raman spectra in the manner in which the spectra are generated. IR spectrum is generated due to a change in dipole moment or charge distribution associated with the vibrational mode of a molecule while scattering causes a momentary distortion of the electrons distributed around a bond in a molecule resulting in temporary polarization of the molecule, pursued by re-emission of the radiation as the bond returns to its normal state. Thus, the molecule becomes an induced dipole moment that disappears upon relaxation and remission. Thus, Raman intensity or activity of a peak depends on the polarizability of the molecule, concentration of the active group and the intensity of the source. Raman spectra detect and display the inelastically scattered light that is energetically downshifted by the characteristic vibrational frequency of a molecule[53].

Docking with human serum albumin

Human serum albumin (HSA) is an important plasma protein with essential functions such as acting as repository and carrier for many exogenous (drugs, nutrients, etc.) and endogenous (fatty acids, bilirubin, etc.) substances in the blood[54]. Thus, it attracts pharmaceutical industries as a better drug delivery system with improved targeting and lower side effects[55]. The pharmacokinetic and dynamic properties of any drug/functional food metabolites like flavonoids[56] are highly influenced by their binding to this protein[57]. Furthermore, albumin influences the rheological properties of erythrocytes[58] by affecting erythrocyte sedimentation and aggregation. Barreca *et al.*[59] studied the interaction and binding of flavonoids to HSA and how this modified its conformation, stability and resistance against aggregation and oxidative injuries. Due to its extensive conformational adaptability, HSA can be considered as a multimeric protein[60]. The biological significance of the present work lies in understanding the interactions of the studied flavones with HSA, which will be vital for designing flavones-derived

Table 4. Binding energy (BE) and inhibition constant (Ki) of docked proteins with flavones

	OxyHb		DeoxyHb		Albumin	
	BE (kcal/mol)	Ki (μM)	BE (kcal/mol)	Ki (μM)	BE (kcal/mol)	Ki (μM)
Chrysin	–6.4	20.53	–5.5	92.72	–6.53	16.39
Baicalein	–6.62	14.14	–5.26	138.6	–6.59	14.74
Oroxylin A	–6.31	23.52	–5.46	99.03	–6.66	13.2
Hispidulin	–6.02	38.71	–5.63	74.72	–6.75	11.29

drugs in future. The mechanism of interactions of different flavonoids with various hydroxy and methoxy substituents (luteolin, apigenin, acacetin, tricin, 5,3′,4′-trihydroxy-6,7-dimethoxyflavone and 5,7,4′-trihydroxy-6,3′,5′-trimethoxyflavone) and bovine serum albumin (BSA) were also studied. The results showed that the binding constants appeared to correlate positively with the existing number of hydroxy groups, and negatively with the methoxy groups. In addition, hydroxyls on ring B interacted more easily with BSA than those on ring A. Baicalein and its complex with Al(III) were compared for their interactions with HSA[60], and under simulative physiological conditions the binding mode was investigated which might help in understanding of baicalein pharmacokinetics. The HSA structure has only one Trp, located in sub-domain IIA Trp-214[61,62]. Chrysin with the least binding energy does not have Trp-214 as the residue surrounding it within 4 Å vicinity while the other three have Trp-214 as one of the residues, which means that these three flavones interact with sub-domain IIA (Table 3). Hispidulin has the highest number of H-bonds, i.e. five and also binding energy (–6.75 kcal/mol) among all studied flavones (Supplementary Figure 4).

Docking with oxy and deoxyhaemoglobin and superposition of docked proteins

The interactions of ligand with proteins imply changes in the properties of macromolecules that may modify their biological activities and/or conformations and allow them to acquire new and, sometimes, unexpected abilities. Antioxidant activity of flavonoids on glycosylation of haemoglobin was also reported[63]. The superposed docked structures deviate from their position at a right angle in Baicalein and to a much greater angle in Hispidulin (Supplementary Figure 5). Our work aims to open new perspectives as well as comparative aspects reflecting the importance of functional groups for the binding of these flavones with oxy and deoxyHb and shows that the properties of both compounds can be modified after the complex formation, resulting, for instance, in a protein structure much more resistant to oxidation and fibrillation. The order of binding energy of flavones docked with oxy and deoxyHb is given in Table 3, while their values along with inhibition constant are given in Table 4. Baicalein and hispidulin have the highest and lowest binding energy for oxyHb and vice versa for deoxyHb. The lowest docking energy was then used for binding orientation analysis through superposition of docked oxy and deoxyHb of the same flavones.

Conclusion

Flavones, in particular, and flavonoids, in general, along with other secondary metabolites are considered to affect cellular signalling mainly through redox-mediated way and thus, various redox effects are being studied. While studying the structure and correlating it with the activity, we have studied the functional groups responsible and the way they influence the activity on a comparative basis. This is important as till today we are not able to focus and defend biological experimental results on grounds of physical and chemical laws with surety, as we are able to defend physical experimental results. These *in vitro* biological experiments and computational studies will help in bridging the gap between clinical medicine and basic science as far as diagnostic and prognostic utility of redox biomarkers in health and disease are concerned. The importance of the selected flavones from the roots of *O. indicum* requires a clear perception regarding their incorporation in blood, bioavailability and possible location in membranes along with their influence on cell signalling mostly through their redox-mediated approach[64]. The phenolic and methoxy groups on the benzene ring remained and contributed to their antioxidant activity. Thus, baicalein showed the highest electrophilicity, nucleofugality, electrofugality and protected the osmotic fragility of erythrocytes at 100 μg/ml. It also has low HOMO–LUMO energy gap which may be responsible for its maximum activity in NO scavenging assay, ferric reduction, reducing power, total antioxidant capacity as well as in total phenol assay. Hispidulin was found protective against erythrocytes fragility at 100 μg/ml but moderately active for antioxidant assay except DPPH assay which may be due to least electrophilicity, electrofugality, nucleofugality and higher HOMO–LUMO energy gap. The most important aspect revealed in this study was that baicalein and hispidulin have the highest and lowest binding energy for oxyHb and vice versa for deoxyHb which was also reflected in their superposed

docked structures where position of baicalein has not changed so much as that of hispidulin. The ethanolic extract also showed concentration-dependent activity profile and a strong positive correlation amongst TFC and TPC, RP, FRAP, NO; whereas a negative correlation was observed between TFC and DPPH. Total phenolic content showed a positive correlation with RP and FRAP. Overall, baicalein is the best flavone among the studied flavones with respect to antioxidant activity and protection against osmotic stress on human erythrocytes, most probably because of its least HOMO–LUMO energy gap among all.

Conflict of interest: Authors declare that there is no conflict of interest.

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