Molecular interaction of flavonoids with the hen egg white lysozyme using spectroscopic approach

Dissertation II submitted to
Lovely Professional University, India
For the partial fulfillment of the award

of

Master of Science in Chemistry

by

Akreeti Barwal
(Registration No. 11306625)

Under the guidance of

Dr. Atanu Singha Roy

School of Physical Sciences, Department of Chemistry

Lovely Professional University, Punjab 144411, India

May 2015

Certificate

This is to certify that the capstone project entitled '**Molecular interaction of flavonoids with the hen egg white lysozyme using spectroscopic approach**', submitted by Akreeti Barwal to the Lovely Professional University, Punjab, India is a documentation of genuine literature review of coming research work approved under my guidance and is commendable of consideration for the honor of the degree of Master of Science in Chemistry of the University.

Supervisor

Dr. Atanu Singha Roy

Assistant Professor

Declaration

I certify that

- the work enclosed in this thesis is innovative and has been carried out by me under the guidance of my supervisor, Dr. Atanu Singha Roy
- the present work has not been submitted earlier to any other university for any degree
- I have been followed the guiding principle provided by the university in the preparation of the report
- whenever I have used resources (such as data, theoretical representations, any figure, and text) from other sources, I have given due recognition to them by citing them in the report and providing their details in the bibliography.

_____

Akreeti Barwal

Date:

Acknowledgement

It is my great pleasure to present the project report on "**Molecular interaction of flavonoids with the hen egg white lysozyme using spectroscopic approach".** Every work skilled is pleasure wisdom. However a number of people always inspire, disparage and welcome a work with their objective ideas and opinions. I would like to employ this opportunity to thank all, who have helped us to complete this project.

Firstly, I would like to thank Dr. Atanu Singha Roy without whose support this could not be completed. I would like to express thank all people who give their precious time and comment to improve this project. I want to thank my university for providing us with assets.

At last I would like to utter my deep gratitude to our head of department Dr.Ramesh chand Thakur for providing all necessary facilities and encouraging.

Akreeti Barwal

Lovely Professional University, Punjab

CONTENTS

List of Tables

Abstract

Protein-ligand binding plays a decisive role in various biological pathways starting from metabolic catalysis to directive of multifarious signals. Interactions of different ligand molecule with biological carrier proteins have important implications for drug delivery. The protein-ligand complexes at atomic level make it possible to design a drug for treatment of diseases. Signal transduction, transcription, translation and enzyme catalysis all are dependent on various kinds of protein-ligand binding processes which are essential in living system. The structure activity relationship, specific modes of binding and corresponding disposal at targeted sites through the carrier protein is essential in field of drug delivery.

The binding of fisetin and morin with hen egg white lysozyme (HEWL) have been carried out by UV-vis spectroscopy and molecular docking studies. The binding affinity of fisetin (3.78×10^{-4} M^{-1} at pH 7.2) towards HEWL is greater than morin (2.33×10^{-4} M^{-1} at pH 7.2). The interaction of polyphenols with HEWL has shown the involvement of electrostatic forces (the binding affinities decreased in presence of higher ionic strength of the media). The binding constants of these polyphenols are estimated at different pH conditions (pH 2.0, 4.3, 7.2 and 9.0) and it has been observed that at pH 2.0, the ligands showed no binding towards HEWL. The highest binding affinity of the ligands has been found in the pH 9.0.

Furthermore we have performed molecular docking studies using *ArgusLab* software to find out the exact position location of these ligands inside the HEWL geometry after binding. It has been observed that both ligands binds near to Trp 62 and Trp 63 of HEWL. The binding free energy of the fisetin towards HEWL (-6.15058 kcal/mol) is found greater than morin (-5.86477 kcal/mol).

1. INTRODUCTION

1.1 PROTEIN-LIGAND INTERACTION: BACKGROUND OF THE STUDY

Protein-ligand binding plays a decisive role in various biological pathways starting from metabolic catalysis to directive of multifarious signals. Protein-ligand and protein-protein interactions are of fundamental importance to processes occurring within living organisms. Interactions of different ligand molecule with biological carrier proteins have important implications for drug delivery. The protein-ligand complexes at atomic level make it possible to design a drug for treatment of diseases. Signal transduction, transcription, translation and enzyme catalysis all are dependent on various kinds of protein-ligand binding processes which are essential in living system. The structure activity relationship, specific modes of binding and corresponding disposal at targeted sites through the carrier protein is essential in field of drug delivery. Selective molecular recognition between ligand and a receptor via non-bonding interaction depends on several factors such as (1) similarities of molecular surfaces, (2) energetically favorable binding and (3) different non-covalent forces acting between the protein and ligand. In study of biomacromolecules there exist several types of binding such as allosteric binding and competitive binding. Allosteric binding is defined by binding of two ligands to two different binding sites. Competitive binding is when two different molecules compete for the same binding site due to structural similarities.

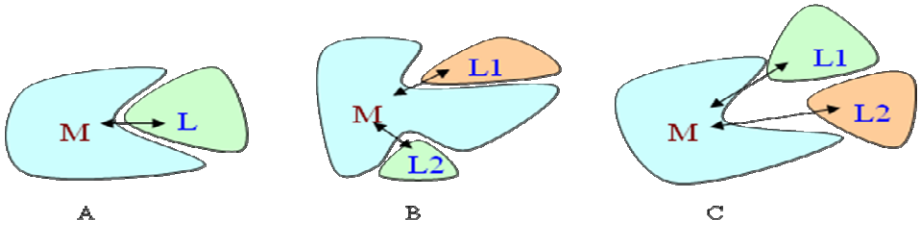


Figure 1. (A) Single site binding; (B) Allosteric binding ; (C) Competitive binding

The following points to be considered while studying macromolecule ligand binding.

- Cooperativity is important in regulation of biological functions.
  - ***Positive cooperativity****:* when one molecule of substrate of ligand increases the affinity of protein for other molecule of different substrate.
  - ***Negative cooperativity****:* when one molecule of substrate decreases the affinity of protein for other molecule of substrate.
- Equilibrium constant of protein-ligand interaction provide thermodynamic measurement of strength of interactions.
- Signal transmission is important to all life processes.
- Protein-ligand interaction is a spontaneous process. Weak interaction leads to strong affinity.

In biological systems, non–covalent connections play a vital role in structural build up of macromolecules. There exist several weak non-covalent forces such as: hydrogen bonding, van der Waals contact, ionic interaction and hydrophobic associations.

i. Non-covalent and reversible binding.
ii. Binding occurs depending on the ligand molecules.
iii. Binding occurs within a particular site of the receptor.
iv. Weak binding forces are usually liable.

Electrochemical techniques are useful for the study of the interaction of micro- molecules with biomolecules and have been extensively used to study the binding reaction of DNA with drugs and metal complexes [1,2] .

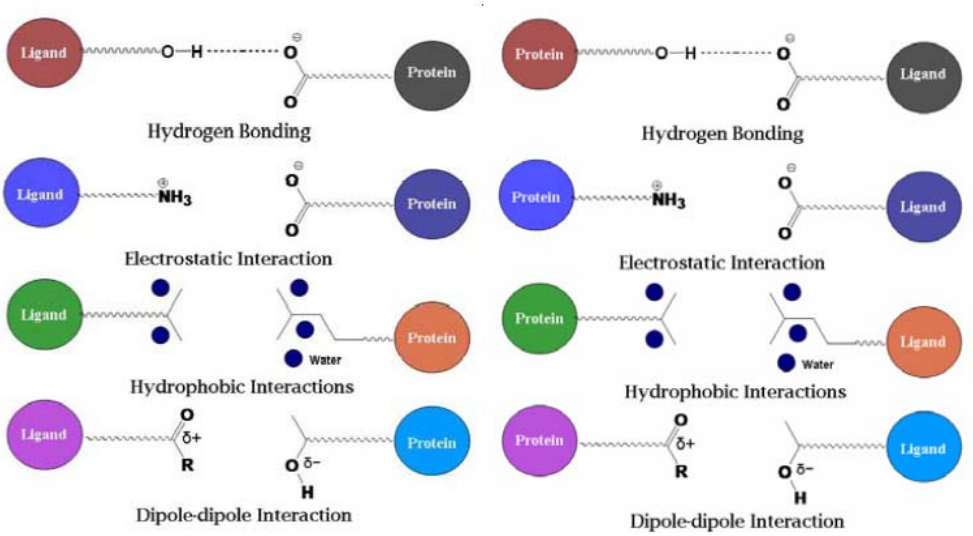


Figure 2. Involvement of weak non-covalent interactions in the biological pathways

The study of lysozyme is important in various biological activities. The interaction of lysozyme with flavonoid is useful in different fields such as medicinal science; pharmaceutical. The binding of lysozyme with flavonoid is examined by UV-vis study, fluorescence spectroscopy, molecular docking, and circular dichroism. Lysozyme has capacity to carry drug so, it has important role in medicinal chemistry. Hence study of protein-ligand interaction is very important. The contact of small medicine like molecules with the organic targets under physiological conditions finds significant applications in the field of pharmaceutical and biomedical research for drug delivery. To examine the interaction among protein and drug is helpful in studying the pharmacological reaction of drugs and design of dosage forms and therefore has become an important in research field [3-6]. Protein be an essential life constituents in the living things, which creates a essential role in life growth and development. Studies on the change in structure of proteins and their binding mechanism with micro molecules have attracted large interest of people, particularly those who were doing researches in life science, chemistry, pharmacy, and clinical medicine. The effective antioxidant and anticancer activities of

polyphenols have attracted scientists in both therapeutic and pharmaceutical fields in recent times. Flavonoids, a assembly of important polyphenols that occur universally in foods of plant source, have been proved to show extensive pharmaceutical activities such as anti- proliferative, anti-tumour, anti-flammation, allergies and so on [7,8]. The properties of flavonoids have been exclusively explained by their bindings to or interferences with enzymes, receptors, transporters and signal transduction system [9]. In this case, it is very useful to study the interaction of flavoniods and proteins for understanding their effects.

Several biophysical methods are useful for the investigation of protein-ligand interactions. Different biophysical methods and their applications is planned in the following Table 1.

Table 1. Different biophysical methods and their application

Methods	Applications
UV-vis study	Determination of ground state association constants
Fluorescence spectroscopy	Determination of quenching, binding, thermodynamic and energy transfer parameters
Isothermal calorimetry	Determination of binding and thermodynamic parameters
Differential scanning calorimetry	Protein stability and folding related to ligand binding
Capillary electrophoresis	Determination of binding affinity
Circular dichroism	Determination of structural changes of the macromolecules after ligand binding
Fourier transform infrared	Determination of structural changes of the macromolecules after ligand binding
Raman spectroscopy	Determination of structural changes of the macromolecules after ligand binding
MALDI mass spectrometry	Determination of mass of the non-covalent complex formed after ligand binding
Nuclear Magnetic Resonance	Determination of the dissociation constant and stoichiometry
Molecular docking study	To find a suitable location of ligand in macromolecule
X-ray diffraction	Determination of crystal structure of the protein-ligand complex

1.2. Factors Affecting on Protein-Ligand Interactions

1.2.1. Chemical Structure

Protein-ligand interactions are greatly influenced by the chemical structure of polyphenols (ligand). In general, high molecular weight and flexibility of polyphenols increase the affinity for complexing proteins [10,11]. B-ring hydroxylation of flavonols has a considerable effect on protein affinity; the more hydroxyl (OH) groups on the B-ring, the stronger binding between protein and polyphenols [12].

Polyphenols such as rutin, baicalin, quercetin, daidzin, puerarin, and genistin (monoglyscosides) has lower affinity for BSA by 5-10 and 5600 times compared with flavonoid (aglycones) baicalein, quercetin, daidzein, and genistein.

Protein structures also have an effect on protein-phenolic interactions. Protein binding to flavanoids can be affected by protein conformation, charge and size of protein, and amino acid composition of protein. [13.14,15].

1.2.2. pH and Ionic Strength

Protein-phenolic interactions are dependent on pH; maximum level of the interactions is at or close to the protein isoelectric point (pI).The binding of polyphenols (quercetin, rutin, and isoquercetin) to other proteins (HSA, BSA, albumin and lysozyme) was decreased by lowering pH [16].

Ionic strength also influences protein-phenolic interactions. Increases in ionic strength diminish the aggregation and precipitation of proteins (α-amylase and BSA) by tannins [17].

1.2.3. Temperature

Increase in temperature leads to decrease in the binding of polyphenols to proteins. Rises in temperature up to 60 ^{0C} and 90 ^{0}C lowered the ability of BSA to complex chlorogenic acid and quercetin, respectively [15,16].

1.3. THERMODYNAMIC ASPECTS OF THE INTERACTION

Molecular recognition is complex in nature and binding processes are oftentimes associated with structural changes of the receptors. Binding affinity (K_b) indicates the strength of the complex produced between a ligand and a receptor. Higher affinity indicates, greater residence time of the ligand bound to the receptor compared to a case of lower binding affinity. The binding affinity, site selectivity and thermodynamic parameters of the protein-ligand complexes are used as an important basis for (QSAR) quantitative structure activity relationship studies. The binding constant (K_b) of a small molecule interacting with a macromolecule can be determined by the estimation of equilibrium constant of binding (K_{eq}) or in terms of dissociation (K_d) over a concentration range at a particular temperature. The following equations can be used to present the equilibrium of the protein-ligand interaction and the equilibrium binding constant.

Protein +Ligand<=> Protein-ligand

$$K_{eq} = \frac{k_f}{k_d} = K_b = \frac{[PL]}{[P][L]}$$

Thermodynamic aspects of the interactions are an important part of binding that provides information on contributions of individual thermodynamic parameter resembling change in enthalpy ($\Delta H°$), randomness ($\Delta S°$) and free energy ($\Delta G°$) accompanying the interaction. The change in the free energy associated with the binding process is related to the equilibrium binding constant (K_{eq}) or binding constant (K_b) as described by equation (*1*). The relation of free

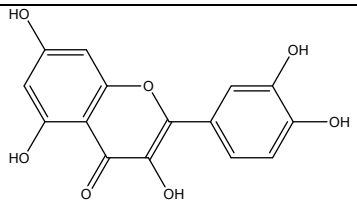
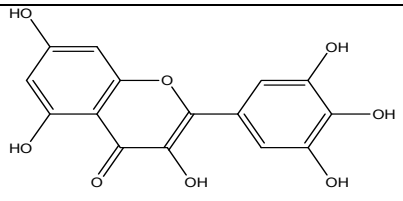
energy change associated with other thermodynamic parameters is presented by equation (*1* and *2*).

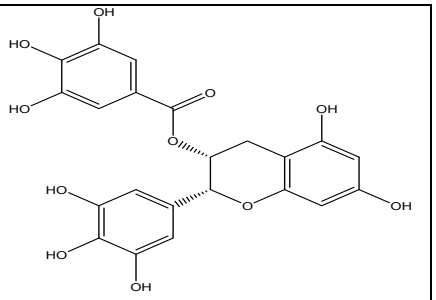
$$\Delta G° = -RT \ln K = -RT \ln K \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

$$\Delta G° = \Delta H° - T\Delta S° \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

The sign and magnitude of the different thermodynamic parameter coupled with various types of interactions that participate in the protein binding procedure have been characterized [18]. Both the high values of $\Delta H°$ and $\Delta S°$ are the consequence of hydrophobic association and low $\Delta H°$ and $\Delta S°$ values are related to the H-bonding and weak forces. The extremely small positive or a negative $\Delta H°$ and positive $\Delta S°$ values be correlated with the presence of electrostatic interactions and hydrophobic involvement that outcomes a positive $\Delta S°$. From the thermodynamic point of view, there occur significant amount of enthalpic and entropic contributions to the overall free energy change of binding during the molecular recognition [19]. Biophysical methods are useful for the investigation of protein-ligand interactions. Some methods and their implications are provided in Table 1.

Table2. Thermodynamic parameter of some Lysozyme-flavonoid systems

SYSTEM	ΔH° (KJ/mol)	ΔS° (J/mol/K)	ΔG° (KJ/mol)	STRUCTURE OF LIGANDS
Lyz-quercetin	-7.558	61.07	-25.15	
Lyz-Myricetin	21.25	183.28	-	

Lyz-EGCG	-27.12	1.47	-27.57	

2. LITERATURE REVIEW

An outline of existing literature available on the structural and binding aspects of lysozyme. The study of binding of different flavonoids (e.g. morin, fisetin and hesperedin) with lysozyme will be performed by fluorescence spectroscopy. The numbers of H-bond donors, dipole moment and molecular molar refractivity make more contribution to the interaction mechanism and binding capacities that's depend upon position of hydrogen and glycosyl position [20]. The binding of drugs to proteins take part in pharmacokinetics of drugs. The pharmacodynamics of a particular drug depends on its interaction with carrier proteins. The contact between flavonoids and lysozyme was examined by absorption and fluorescence spectroscopy. The binding constants, no. of sites for binding, fluorescence anisotropy, thermodynamic parameters and energy transfer mechanism be also investigated. Conformational changes in proteins was also observed from the synchronal, three dimensional and circular dichroism. In protein-ligand both the hydrophobic and ionic interactions involves in stabilization of complex. [21].In myricetin and lysozyme interaction, binding constant decreases on decrease in the pH from 7.4-6.7 or increasing from 8.0-7.4 because both bears same charge i.e. ionic repulsion at pH 6.7, which may be liable for decrease in binding ability and increase in binding constant should recognized by N-B transition in protein [22]. The structural characteristics of flavonoids with lysozyme be probed, binding affinities will be determined over different pH conditions. Due to alteration in protein structure ,

the binding affinity of drug to protein increased in lower pH conditions. The pH influence the change greatly, fluorescence and circular dichroism shows a large change, at higher pH.The substituent's of flavonoids had influence on the magnitude of interaction.

In a fixed concentration of lysozyme different amount of chloramphenicol was added, fluorescence intensity decreases with no shift that indicate that chloramphenical might show small interaction with lysozyme and quenches its fluorescence [23].The intrinsic fluorescence emission of lysozyme originates from Trp 62 and 108 [24].

The environment in the region of protein was examined by fluorescence, and CD spectrometric technique. The results obtained from these spectra imitate the atmosphere around chromospheres molecules. Fluorescence spectrometric technique used to examine the environment around amino acid residues. The shift in wavelength of emission maximum is associated with the change in the polarity around the chromophore molecule. When the excitation and emission wavelength was stabilized at 15 nm, the fluorescence gave sequence of tyrosine residues. And when it was stabilized at 60nm, the spectral feature of amino acid i.e. tryptophan residues was obtained [25].

Increase in temperature leads to decrease in the stability of the complex results in reduction of binding constant. In case of the dynamic quenching high temperatures can cause increase in collision and leads to increase in quenching constant.

The UV–vis spectra of morin changes from acidic to basic pH condition. At slightly acidic pH, morin show a maximum at 370nm (in ethanol), which is red shifted to 387 and 390 at pH 7 and 9 respectively. This implies that morin is deprotonated at basic pH. The 4′-OH position in polyphenol is in conjugation with B-ring and the C-ring. Therefore, high possibility of deprotonation occurs at pH condition higher than its pKa1 value. In case of morin there is no intramolecular hydrogen bonding between 2′-OH and4′-OH that leads to dissociation of 4′-OH

under the experimental conditions [26]. At basic pH, a new peak is generated in the region of 300 nm due to dissociation of 7-OH (bandII) occurs. Consequently, morin exists as anionic species in solution due to deprotonation of the phenolic OH groups.

The intrinsic absorption peak of tryptophan (Trp) and tyrosines (Tyr) residues in lysozyme (π-π*) gives a absorption bands at 280 nm .Chicken egg white involve many amino acid residues which are appropriate for this study.

Solubility of polyphenols depends on various factors such as chemical structure, the nature of the solvent used in extraction of polyphenols, pH and temperature. The solubility of flavonoids such as quercetin, isoquercitin, rutin, and hesperetin in acetonitrile, acetone, and tert-amyl alcohol was investigated. Quercetin shows high solubility in acetone. Hesperetin and naringenin shows highest solubility in acetonitrile. In some flavanoids water is considered as a weak solvent.

2.1. LYSOZYME: BIOLOGICAL TARGET

Lysozyme is known as N-acetylmuramide glycanhydrolase, or muramidase [27]. These are enzymes which involves the hydrolysis of 1,4-β-linkages connecting N-acetylmuramic acid and N-acetyl-D-glucosamine moiety**.** Lysozyme is an antimicrobial proteinase. Lysozyme has many physiological as well as pharmaceutical functions. Therefore, studies on the interactions between micro molecules and Lysozyme have a significant importance on realizing the transport and metabolism process of the small molecules .Lysozyme present is high in secretions like [28]:

- Tears, sliva, human milk and mucus
- Present in hen egg white

Hen egg white lysozyme is a 14.6 kDa single chain protein. It contains four disulphide bonds involving eight cysteine residues, six tryptophan's (Trp) and three tyrosine's (Tyr). Disulphide bonds stabilize the tertiary structure of protein. Lysozyme is a protein that contains 129 amino

acid and important due to its pharmological activity. As the protein rotates there become deep clefts where six carbohydrate molecules can bind. Lysozyme has five helical regions in the whole structure. Three are standard alpha helices and five regions of beta sheet which have aptitude to coil and turn randomly. Active place for binding in lysozyme are in the close vicinity of Trp 62 and Trp 63.

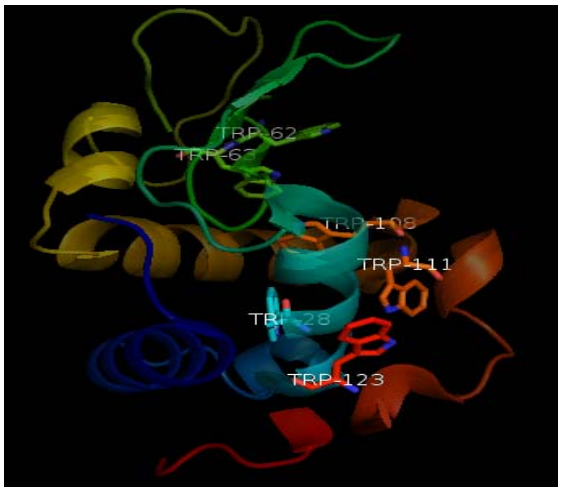


Figure 2: Representation of hen egg white lysozyme (PDB ID: 3rz4)

Lysozyme protects us from several bacterial infections [29]. It also acts as enzyme that catalyzes the protection of cell walls of bacteria. Bacteria have a tough coat of carbohydrates, which are sheltered by different short peptides. Lysozyme breaks down this carbohydrate sheet, destroying the integrity of the cell wall and as a result of it, the bacteria died under their own internal pressure. In blood, lysozyme provides shield along with the methods employed by the immune system.It has anti-viral, anti-inflamatory, anti-histaminic, anti-tumor actions [30]. Lysozyme is used in food industry to prevent spoilage of food. Lysozyme be used in pharmaceutical field. Lysozyme helps to fight against bacterial infections. Hen egg white lysozyme retains a structure which is highly homologous to human lysozyme and responsible for the formation of systemic amyloidosis in the human body.

2.2 FLAVONOIDS: BIOLOGICAL ASPECT

Polyphenols have been studied widely for their multifarious biological activities. Flavonoids are important dietary polyphenols. Flavonoids are low molecular weight components. They consists of two aromatic rings A and B attached by three carbon atoms represent the chief and most varied family of polyphenols characterized by the diphenylpropane skeleton (C6C3C6), consisting of two aromatic rings connected by three carbons forming a heterocyclic ring. They are abundant in various natural food components such as tea, coffee, fruits, vegetables, beans, chocolates, wines etc. They are a category of plant derived secondary metabolites. Flavonoids are separated and identified from various plants and classified to flavones, flavonol, and isoflavone according to different structures:

Flavonols:

Kaempferol, quercetin and myricetin represent the high percentage of flavonols in foods .They are commonly present in vegetables, fruits and beverages. The richest sources of flavonols are onions, tomato skin, curly kale, leeks and broccoli [31, 32].

Flavones:

Flavones consist of glycosides of luteolin and apigenin which are present in sweet red pepper and celery. Skin of citrus fruit and cereals such as millet and wheat also include larger amount of flavones [33, 34, 35].

Isoflavones:

Soybean and its related products have a high concentration of the three isoflavones genistein, daidzein, and glycitein.

The structural differences also affect their binding process with plasma proteins. The interaction involving in flavonoids and proteins has attracted great interest among researchers [36-45]. The

interaction between flavonoids and proteins results to form stable complexes. The dietary polyphenols exist in nature as β-glycosides. Glycosides hydrolyzed to their aglycones to generate effect on body. Flavonoids were designated to as vitamin P (due to their permeability nature of vascular capillaries). The essential structure of flavonoids allows a exchange patterns: phenyl hydroxyl, o-sugar, methoxy groups [46]. Flavonoids have been considered widely for their various biological aspects. The UV-vis spectra of flavonoid display two types of absorptions, (i) at (240-280 nm) and (ii) (300-400 nm) for the benzoyl and cinnamoyl moieties respectively [47]. The antioxidant efficacy associated with polyphenols (flavonoids) is mainly due to their free radical scavenging properties. The B-ring of polyphenols is proposed to be the major site of antioxidant reactions. The lipophilicity of flavonoid group has expressed in terms of a partition coefficient (log P) with values obtained from different literatures [48][49][50].

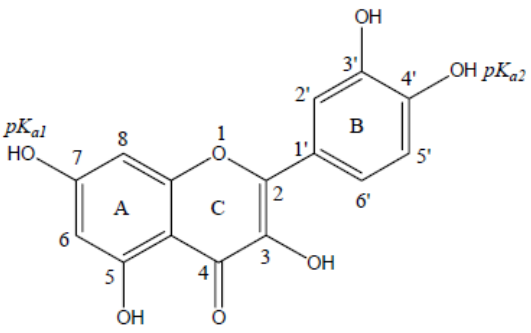
Flavonoids comprises of biological and pharmacological activities. Anti-inflammatory, anti-fungal, anti-bacterial, anti-cancer activities [51][52]. Anti-oxidants are a compound that protects the cells beside the harmful cause of reactive oxygen such as hyperoxide, native oxygen, and hydroxyl radical. The capacity of flavonoids to acts as antioxidant depends upon molecular structure. Polyphenols are linked to protective effects against cardiovascular disease and different forms of cancer. Polyphenols can be used as colorants and preservatives in food. Polyphenols also improve the foaming properties of both β-lacto globulin and egg white proteins.

Table 3. Structural position of different functional moieties

Category	R_3	R_4	R_5	R_6	R_7	R_2'	R_3'	R_4'
Morin (Flavonol)	OH	O	OH	H	OH	OH	H	OH
Rutin (Flavone)	$OC_6H_{12}O_6$	O	OH	H	OH	H	OH	OH
Fisetin (Flavone)	OH	O	H	H	OH	H	H	OH
Genistein (isoflavones)	C_6H_5OH	O	OH	H	OH	H	H	OH
Hespertin (Flavanone)	H	O	OH	H	OH	H	OH	OCH_3
Myricetin (Flavanol)	OH	O	OH	H	OH	H	OH	OH

Quercetin

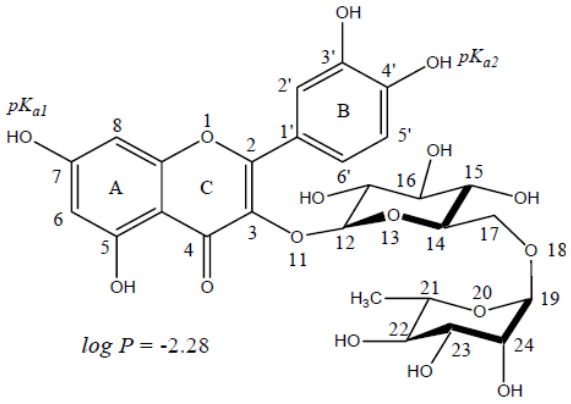
3,5,7,3′,4′-pentahydroxyflavone is a plant consequent anti-oxidant dietary polyphenol, used as a dietary complement. *In vitro*, it shows anti-tumor, anti-inflammatory and anti-cancer actions [53]. In a



log P = 2.74

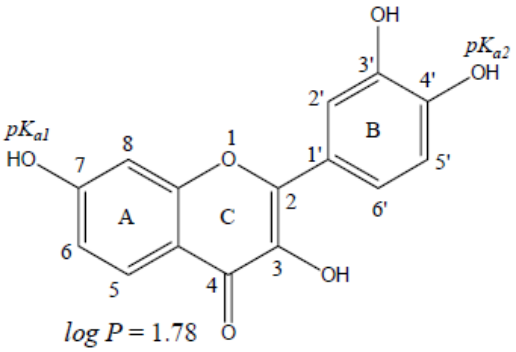
recent study, quercetin was found to be effectual in the diminution of liver damage in mice model [54].

Rutin



3,3′,4′,5,7-pentahydroxyflavone-3-rutinoside is called rutoside or quercetin-3-rutinoside, is a citrus flavonoid . Rutin is a useful agent against the human follicular dermal papilla cells (HFDPC) apoptosis that is one of the most important agents of hair bulb regression [55]. It has also been found to be effective in the lessening of oxidative stress and is able to restrain nitric oxide synthase activity apart from the ability to reduce the possibility of mitochondrial scratch [56].

Fisetin



Like quercetin, fisetin (3,7,3′,4′-tetrahydroxyflavone) exhibits the anti-oxidant, anti-inflammatory [61] and anti-carcinogenic activities [57][58] in different cell lines. The molecule is also indispensable for its capacity of reducing kidney failure in diabetics [59]. Another recent study has verified that the nanoemulsion formulation of fisetin is capable to increase bioavailability and anti-tumor activity in mice model [60].

Morin

3,5,7,2′,4′-pentahydroxyflavone is derived from the *Maclura tinctoria* (old fustic), *Maclura pomifera* (Osage orange) and from the leaves of *Psidium guajava* (common guava),

demonstrates the potential as a multi-functioning drug molecules [61]. Morin is also initiates to diminish free radical induced to break the cardiovascular cells [62]. A recent study showed that the flavonol is capable to reduce the oxidative stress induced by high glucose content in rat hepatocytes [63]. Morin is also found to function by the commencement or structural alteration of other enzymes [64][65], hence it's binding with carrier proteins is important from a biological application point of view.

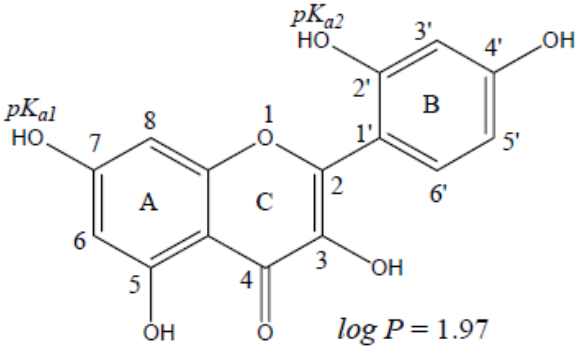


Table 4. log *P* and Dipole moment of Flavonoids

Flavonoids	**log *P***	**Dipole moment**
Quercetin	0.35	5.183
Baicalein	0.02	5.726
Rutin	-2.28	4.388
Genistein	1.74	3.627
Naringin	-0.49	2.991

3. SCOPE OF THE STUDY: PROTEIN-LIGAND BINDING

Studies on the interaction between micro molecules and lysozyme are significant on realizing the drug transport and metabolism process. Protein-ligand binding have developed an exhilarating research ground in chemistry, biology and pharmaceutical sciences. Protein-drug interaction plays an imperative role in various biological processes within living systems. The circulation

and metabolism of several biological active compounds in the body are directly interconnected with their relative affinities towards the biological targets e.g. proteins and DNA. The investigation of binding of such molecules to the carrier protein, lysozyme is of indispensable and essential importance. Understanding of interactions involved in drug binding at molecular level to different targets is mandatory in the field of pharmacology and pharmacokinetics due to possible toxicological aspects of the ligand molecules [66]. The interaction is crucial for the development of molecules, useful for therapeutics. Several biophysical techniques have been employed to investigate the interactions at molecular level. For hen egg white lysozyme, in acidic and neutral solution, activity of lysozyme is initiated to be extra in acidic solution than the activity of lysozyme in neutral solution. The optimum pH for lysozyme activity is 6.2 and maximum lysis was set to take place between pH 6.0-7.0 [67]. Recently few literatures have published regarding the binding of small dietary flavonoids with lysozyme. In the current study, we have planned to execute the interactions of fisetin and morin with lysozyme under different pH conditions. The binding is very essential in context of drug delivery and development and different pH conditions are important to investigate the structural changes of both protein and ligand molecules.

4. OBJECTIVE AND WORK PLAN

1) To study the interaction of dietary polyphenols with the hen egg white lysozyme using spectroscopic approaches such as spectrophotometry, fluorescence anisotropy and circular dichroism measurements
2) To evaluate the binding parameters for the interactions at different pH conditions from UV-vis studies
3) To investigate the secondary structural changes of the protein during binding with the ligands
4) To substantiate the experimental findings with the help of the molecular docking study

5. MATERIALS AND RESEARCH METHODOLOGY

5.1 Materials

Chicken egg lysozyme (MB098) and morin hydrate (RM1130) were taken from Himedia Laboratories, India. The flavonoid fisetin (T0121) was purchased from TCI Chemicals, India. Other analytical grade chemicals were obtained from Loba Chemie Pvt. Ltd. India. The lysozyme was dissolved in 20 mM phosphate buffer of pH 2,4.3,7.2,9.0 and the concentration was measured spectrophotometrically (Shimadzu, model UVPC 1800) using ε_{280} = 37646 M^{-1} cm^{-1} . The flavonoids were dissolved in absolute grade ethanol.

5.2 RESEARCH METHODOLOGY

The interactions of flavonoids with lysozyme will be investigated in details using standard biophysical techniques. Spectroscopic techniques e.g. steady-state fluorescence and UV-vis will be used for the determination of binding constant and number of binding sites. The changes in secondary structures of lysozyme are to be determined from circular dichorism (CD).

5.2.1. UV-vis study

UV-vis studies were conducted on a Shimadzu (model UVPC 1800) spectrophotometer in the region of 200-600 nm using a 1 cm quartz cuvette in 20 mM PB of pH 2.0, 4.3, 7.2, 9.0 at room temperature. Under different pH conditions a sample of 25 μM fisetin (or morin) was titrated with consecutive addition of the protein (0 to 30 μM). UV-vis spectroscopy (absorption spectroscopy) molecules consisting of electrons in bonding and non-bonding that can absorb energy in form of UV-vis region to stimulate these electrons to higher anti-bonding orbitals.The gap between HOMO and LUMO is responsible for absorption of light at different wavelengths.This technique is used for the determination of the ground state association constant. With the help of Benesi-Hildebrand equation [68] we can estimate the binding affinity for the 1:1 ground state complexation between ligand and the protein.

$$\frac{1}{\Delta A} = \frac{1}{(\varepsilon_b - \varepsilon_f)L_T} + \frac{1}{(\varepsilon_b - \varepsilon_f)L_T K_a}\frac{1}{M} \qquad (1)$$

where ε ; extinction coefficient and M ; concentration of the protein. The subscripts b, f and T are known as bound, free and total ligand. L_T ; the total ligand concentration and ΔA the change in the absorbance at a particular wavelength. The plot of the reciprocal of ΔA versus the reciprocal of concentration of lysozyme will provide the value of K_a from the ratio of intercept to slope. The individual spectra were checked by subtracting spectra of protein from the protein-ligand collective spectra.

5.2.2. Fluorescence anisotropy

Fluorescence anisotropy (polarization) provides information about the interaction between small molecule and biomacromolecules. The binding of micro molecules with biomacromolecules restricts the motions of the small molecules and it gets organized in a particular direction of the

applied polarization which leads to the increase in rotational correlation time as well as anisotropy of the system. The steady-state anisotropic dimensions were carried out to obtain the anisotropy (*r*). The anisotropy measurements were performed on a Fluoromax 4 spectrofluorometer from Horiba Jobin Yvon. The excitation and emission wavelengths of morin (20 μM) and fisetin (20 μM) were fixed at 390/517 and 370/535 nm respectively keeping 10/10 nm slit widths.

5.2.3. Circular dichroism study

CD studies were conducted on a Jasco-810 spectrophotometer in the region 190-240 nm at room temperature. Two sets of solutions were taken having lysozyme: flavonoid 1:0 and 1:5 respectively. For far UV CD measurement the concentration of lysozyme was stable at 20 μM. The far-UV CD spectrum of the protein was collected using 0.1 cm cell with a scan speed of 50 nm/min. The left and right circularly polarized components may be absorbed to different extents when a linearly polarized light passed through an optically active molecule. The light absorption follows the Beer-Lambert's law and difference between the absorbance results in circular dichorism.CD is important for the structural determination of proteins in folded and unfolded states.Generally,the α-helices are identified by negative bands with distinct similar magnitude at 222 and 208nm due to n-π* and π-π* transistion respectively. The β-sheet contents are characterized by the presence of negative band at about 216nm and a positive band of similar magnitude around 195nm.CD experiments have been carried out on a spectropolarimeter to detect the effect of ligand on the secondary structural organization of the protein. The far-UV CD spectra were normally collected in the range of 190 to 240 nm with the help of a 0.1 cm cell path

length using a scan speed of 50 nm/min. The mean residual ellipticity (*MRE*, in deg cm^2 $dmol^{-1}$) can be estimated from the subsequent equation 4 [70].

$$MRE = \frac{observedCD(m\deg)}{C_p \times n \times l \times 10} \qquad (4)$$

C_p concentration of lysozyme and l ; path length of the cell. From the *MRE* value at 208 nm one can estimate the alpha-helix percentage of free and protein-ligand complex using the following equation 5 [71].

$$\alpha - helix(\%) = \frac{(-MRE_{208} - 4000)}{33000 - 4000} \times 100 \qquad (5)$$

where MRE_{208} is the *MRE* values obtained from CD measurements, 33000 and 4000 are the *MRE* values of a α-helix and that of a β-form and random coil arrangement at 208 nm respectively.

5.2.4. Molecular docking study

The 3D arrangement of lysozyme (PDB ID: 3rz4) is available in the Protein Data Bank. The energy minimized structure of the flavonoids can be created via Argus lab 4.0.1 using Austin model1 (AM) or UFF (universal force field) all based on the neglect of diatomic differential overlap (NDDO) approximate Hamitonian. The gradient convergence used is 10^{-1}Kcai/mol/Ang. Other parameters number of iterations, convergence were fixed at 1000, 10^{-10} Kcal/mol . The docking of fisetin and morin with Lysozyme can be performed by PyMol was used to visualize docked conformations [72]. The grid minimum(X,Y,Z) 15.6675 ,-5,37246 ,-11.5768 and grid maximum (X,Y,Z) 30.6675 ,17.8275 ,5.2232 was fixed for both fisetin and morin.The

hydrophobic constant used having value 0.0373.The ΔG (free energy) and K_b (binding constant) calculated by the dockng.

6. RESULTS AND DISCUSSION

6.1. UV-vis study for fisetin and morin

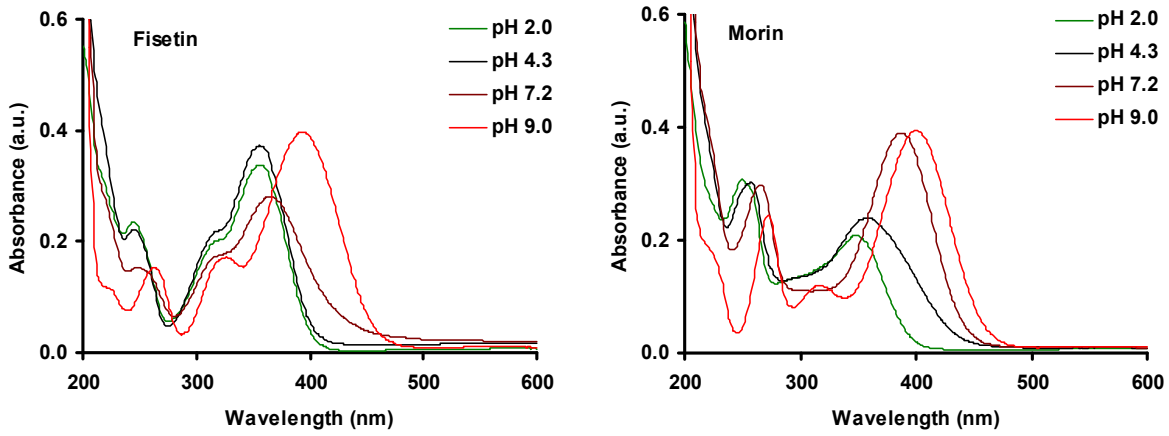


Figure 3. UV-vis spectra of fisetin (25 μM) and morin (25 μM) in different pH conditions

The UV-vis spectra of fisetin in different pH are presented in Figure 3. Figure 3 exhibits that there are three peaks for fisetin at 247, 319 and 360 nm in pH 2.0 and 4.3. But in higher pH (i.e. 9.0), the peaks are shifted to 268, 329 and 398 nm respectively, indicating the formation of new species in the medium.

Similar results are also observed in case of morin. It exhibits two peaks in the UV-vis region at 255 and 343 nm. The increase in pH causes deprotonation in the structure and anionic species are formed even in neutral and also in basic medium. In pH 9.0, the peak positions are shifted to 275 and 404 nm respectively, indicating the presence of some anionic species in the medium. Hence the binding of lysozyme with these ligands in different pH has some importance to acquire some information regarding the structure-activity relationship.

Table 5: ***UV-vis peak positions of fisetin and morin***

Molecule	pH 2.0	pH 4.3	pH 7.2	pH 9.0
FISETIN	247 nm	247 nm	252 nm	268 nm
	319 nm	319 nm	317 nm	329 nm
	360 nm	360 nm	367 nm	398 nm
MORIN	254 nm	261 nm	269 nm	275 nm
	352 nm	359 nm	392 nm	404 nm

6.2. UV-vis study for the binding of flavonoids with lysozyme in different pH

6.2.1. UV-vis study in acidic pH

The UV-vis spectra of fisetin or morin in absence and presence of lysozyme in pH 2.0 (200 mM phosphate buffer) and 4.3 (200 mM phosphate buffer) in 1:1 molar ratio are presented in Figure 4. From Figure 4 (for pH 2.0), it may be concluded that no such significant binding is present between fisetin or morin and lysozyme in pH 2.0 because no such decrease or shift is found in the UV-vis spectra of protein as well as ligands. But in pH 4.3 (Figure 4), the absorbance of fisetin or morin is decreased in presence of lysozyme indicating the presence of specific interaction in the binding. Our next objective is to determine the binding affinity of fisetin or morin in pH 4.3 using the concept of Benesi-Hildebrand.

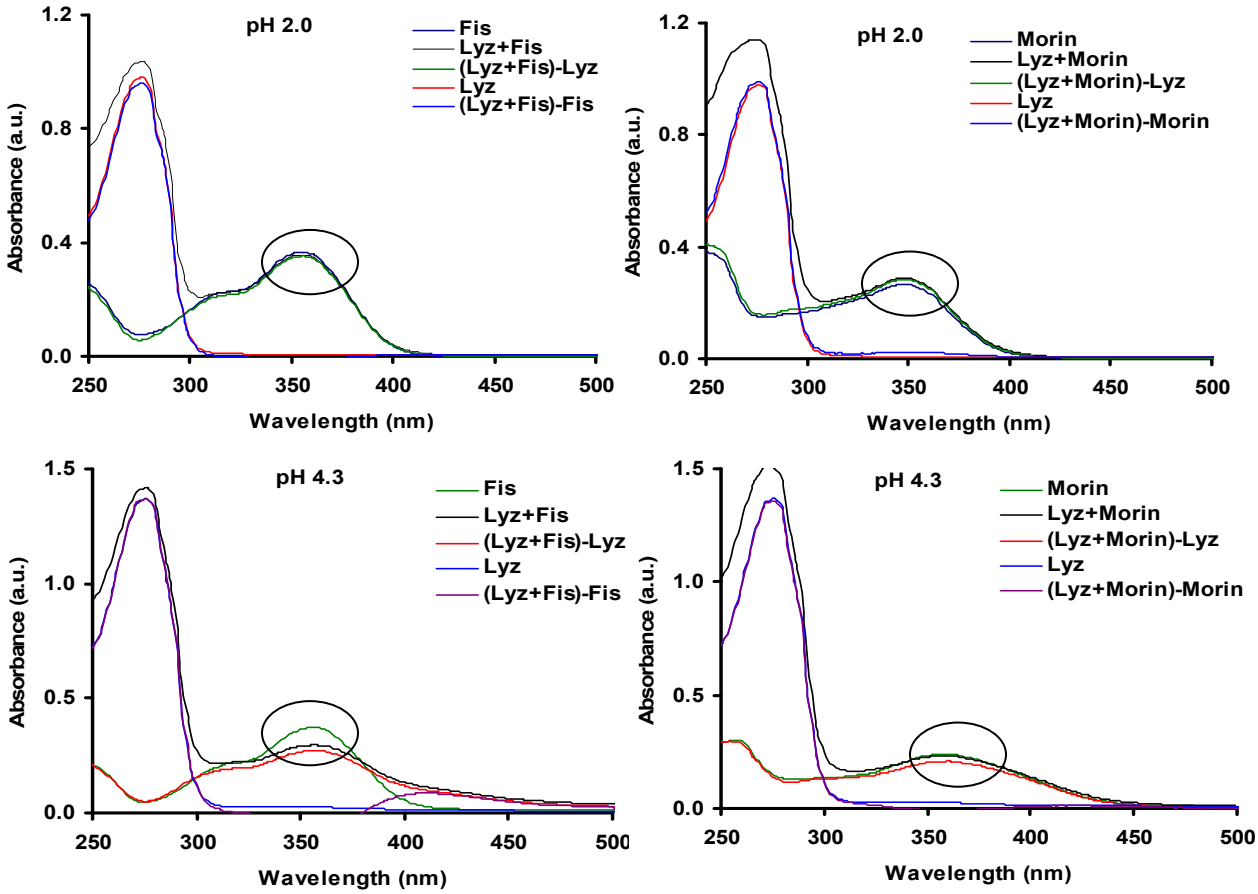


Figure 4. UV-vis spectra of fisetin (25 μM), morin (25 μM), lysozyme (25 μM) and lysozyme-ligand complexes in 200 mM phosphate buffer of pH 2.0 and 4.3.

6.2.2. UV-vis study in pH 7.2

The UV-vis spectrum of flavonoid fisetin consists of three peaks at 250, 314 and 365 nm in pH 7.2 and the peak corresponds to 365 nm is shifted slightly (~ 5 nm) in presence of the protein lysozyme (Figure 5a). In similar experiment with morin, it has been observed that the UV-vis spectrum of species is red shifted from 392 to 400 nm in the presence of protein (Figure 5b). In both the cases the absorbance values at maxima positions are getting reduced with the addition of lysozyme, indicating the creation of complex between protein and ligand. These results suggest that the π–π^* transition of morin and fisetin are getting stabilized. These results indicate the

presence of electrostatic interactions between fisetin or morin and the residues of lysozyme. Similar type of outcomes have been observed in the case of interactions of fisetin and morin with HSA and BSA respectively [73,74].

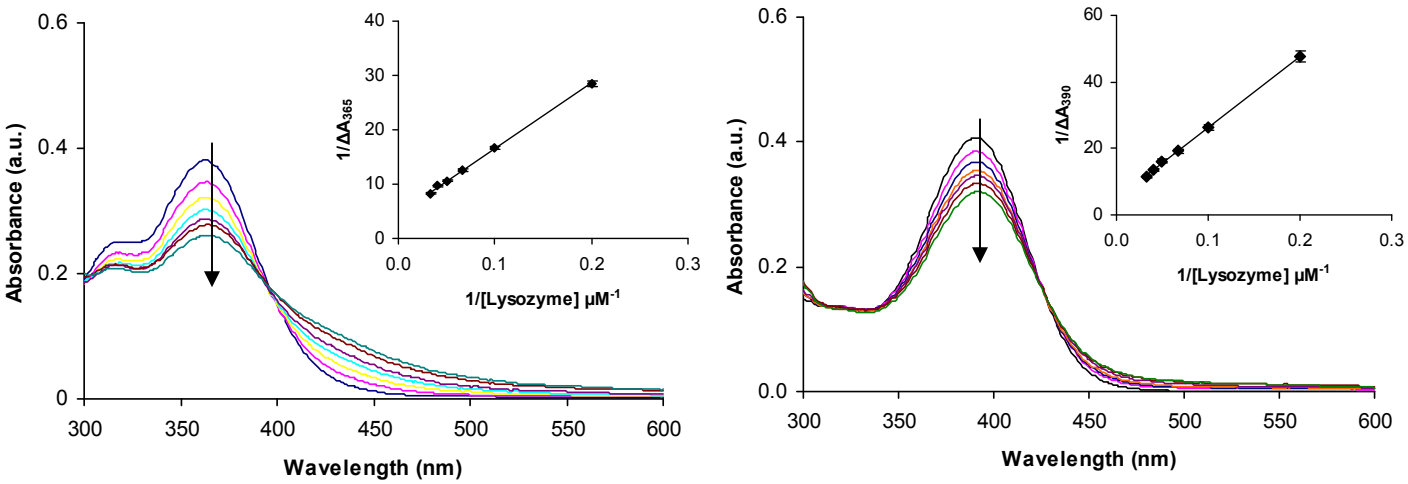


Figure 5. (a) UV-vis spectra of fisetin (b) UV-vis spectra of morin (25 μM) in presence of lysozyme (0 to 30 μM) in 20 mM phosphate buffer of pH 7.2. (a) and (b): corresponding Benesi-Hildebrand plots for the interactions (*J. Incl. Phenom. Macro.* DOI: 10.1007/s10847-014-0465-8)

The ground-state binding constant (K_a) have been estimated using the Benesi-Hildebrand equation (inset of Figure 5a and 5b) and the values are found to be $(3.78 \pm 0.21) \times 10^4$ M^{-1} ($R^2 = 0.99$, for fisetin) and $(2.33 \pm 0.39) \times 10^4$ M^{-1} ($R^2 = 0.99$, for morin).

6.2.3. UV-vis study in pH 9.0

The absorption spectrum of morin in pH 9.0 with the increase in concentration of lysozyme is shown in Figure 6. When lysozyme is added to morin at pH 9.0, the decrease in the absorbance shows that there is a specific interaction taking place among morin and lysozyme. No such

significant spectral shift has been observed and the binding constant for the interaction has been calculated from the Benesi-Hildebrand equation. The ground-state association constant has been found to be $(20.45\pm3.85)\times10^3$ M^{-1} (R^2 = 0.97). It has been experimentally observed that the binding affinity decreased from pH 7.2, indicates the structural importance in the binding phenomena. It may be due to the structural changes of lysozyme or due to the lower binding affinity of anionic species of morin towards the protein.

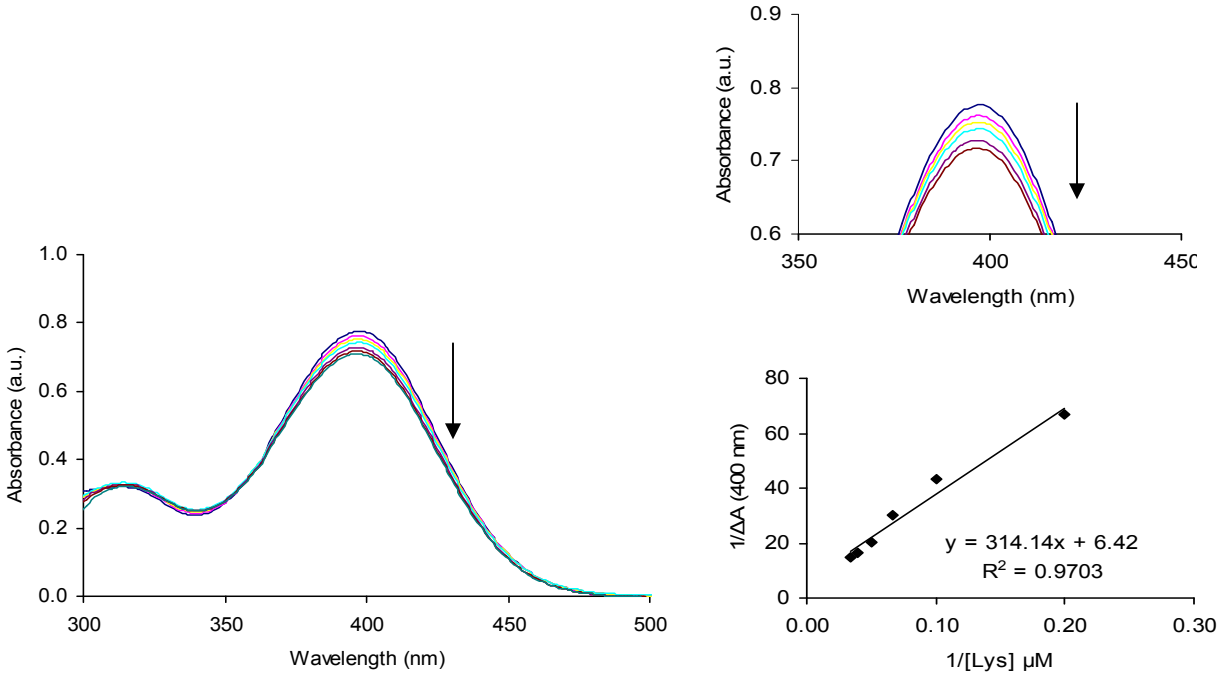


Figure 6. (a) UV-vis spectra of morin (25 µM) in absence and presence of lysozyme (0 to 30 µM) in 200 mM phosphate buffer of pH 9.0. (b) The same spectra in the wavelength region 350-400 nm for better representation and (c) The Benesi-Hildebrand plot for the interaction.

6.2.4. UV-vis study in pH 9.0

The absorption spectrum of fisetin in pH 9.0 with the increase in concentration of lysozyme is shown in Figure 7. When lysozyme is added to fisetin at pH 9.0, the decrease in the absorbance indicates that there is a specific interaction taking place between fisetin and lysozyme.

Significant red shift has been observed indicating some anionic species in medium and the binding constant (K_a) for the interaction has been calculated from the Benesi-Hildebrand equation. The ground-state association constant has been found to be 7.07×10^4 M^{-1} (R^2 = 0.97) which is greater than morin.

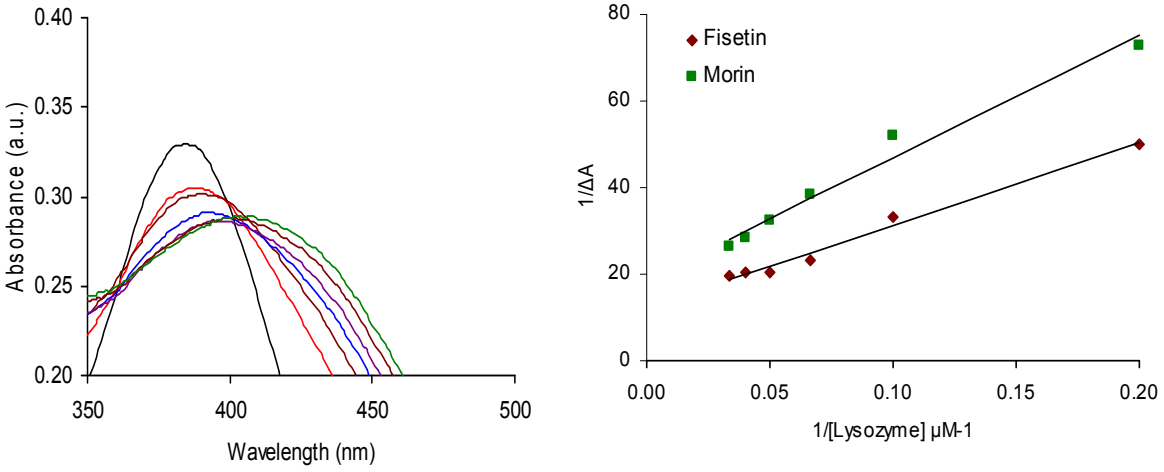


Figure 7. (a) UV-vis spectra of fisetin (25 µM) in presence of lysozyme (0 to 30 µM) in 20 mM phosphate buffer of pH 9.0. (b): corresponding Benesi-Hildebrand plots for the interactions

6.2.5 UV-vis study in pH 4.3

To confirm the structural change of lysozyme by the addition of fisetin and morin, we measured the UV-vis absorption spectra at different concentration of lysozyme (Figure 8). We observed that the absorption peaks at 360 nm decreases evidently and have a reasonable blue shift (about 5 nm) with the increase in the concentration of lysozyme, which indicates that lysozyme molecule contacts with fisetin or morin to form a lysozyme-fisetin (morin) complex. The binding constant for the interaction has been considered from the Benesi-Hildebrand equation. The ground-state association constant has been found to be 1.52×10^{-4} M^{-1}(for fisetin) and 1.04×10^{-4} M^{-1}(morin). It

was found that the binding affinity decreases at acidic pH.

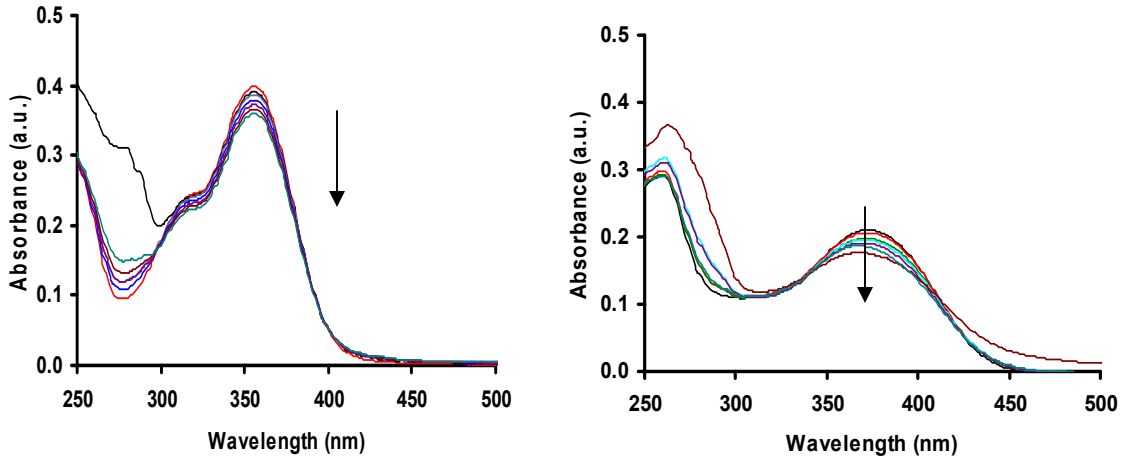


Figure 8: UV-vis spectra of (a) fisetin and (b) morin in absence and presence of HEWL (0 to 30 µM) in 20 mM phosphate buffer of pH 4.3.

From UV-vis studies at different pH conditions (4.3, 7.2 and 9.0) it was clearly shows that a complex formation takes place between protein and ligand and no such type of complexation is take place at pH 2.0.

Table 6: ***Binding constants of fisetin and morin with HEWL***

	pH	*Ionic strength*	*$10^{-4}\times K_b$ (M^{-1})*	*Ionic strength*	*$10^{-4}\times K_b$ (M^{-1})*
LYZ-FISETIN	4.3	0.2 M	-	0.02 M	1.52
	7.2	0.2 M	-	0.02 M	3.78
	9.0	0.2 M	2.01	0.02 M	7.07
LYZ-MORIN	4.3	0.2 M	-	0.02 M	1.05
	7.2	0.2 M	-	0.02 M	2.33
	9.0	0.2	2.05	0.02	6.66

6.3. Fluorescence Anisotropy

The ligand doesn't show such significant anisotropic change with the biomacromolecules. The binding constant of such interactions is not being calculated due to this small change. The molecular docking result also reveals that the ligand (morin or fisetin) is present outside so no such interaction is possible.

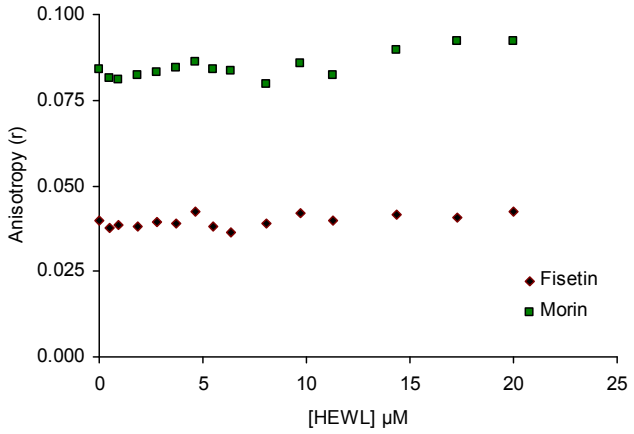


Figure 9: Fluorescence anisotropy of (a) fisetin (20 µM) and (b) morin (20 µM) with lysozyme (0 to 18 µM)

6.4. Circular dichroism results

The CD spectra of lysozyme exhibit two negative bands one around 208 nm ($\pi \rightarrow \pi^*$ transition) and the other one at 222 nm ($n \rightarrow \pi^*$ transition) as presented in Figure 9. It is a characteristic of α-helical pattern originated due to negative cotton effect [75,76]. The complex formation between fisetin (or morin) and lysozyme leads to a small change in spectral intensity of the far UV-CD lacking any considerable shift of peak positions, indicating the increase of helical content after binding [77,78]. It has been found that on binding with fisetin and morin the α-helix percentage of lysozyme enhanced from (31.84±1.59; mean value±se) to (37.81±0.77)% and (37.45±0.53)% at a

molar ratio of 1:5 respectively. The results show the presence of binding between polyphenols and protein, leading to a gain in helical stability of lysozyme (J. Incl. Phenom Macro. DOI:10.1007/S 10847-014-0465-8).

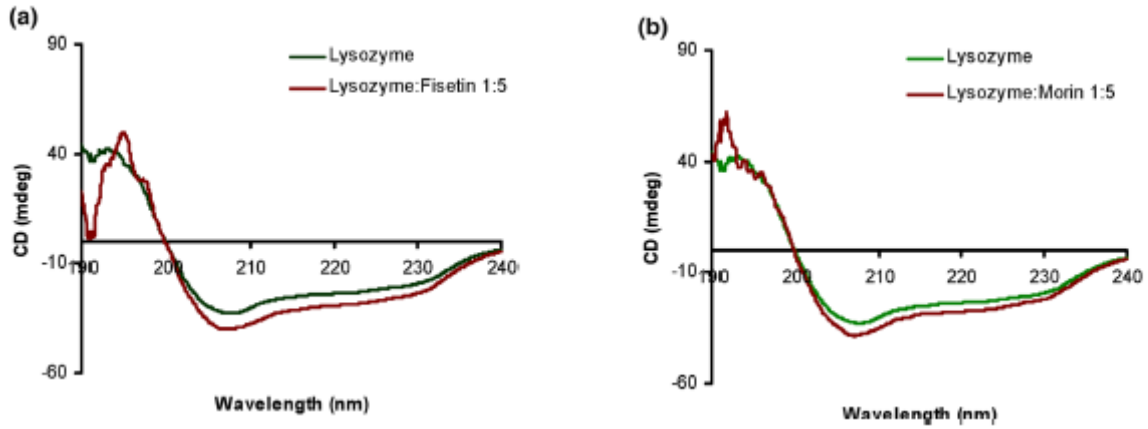


Figure 9: (a) Far UV-CD spectra of lysozyme and its lysozyme-fisetin (1:5) complexes (b) lysozyme-morin (1:5) complexes in 20mM PB of pH 7.2

6.4. Molecular docking results

Molecular docking has been performed to substantiate the investigational data. The stereo view of docked conformation of fisetin (morin) with lysozyme is shown in fig. The geometry optimization of fisetin (morin) is done via. Argus lab 4.0.1 using Austin model 1(AM) or UFF (universal force field).The grid dimensions for ligands (fisetin or morin) is found to be (37×59×43).In case of fisetin ,50 final unique configurations were obtained and 48 unique configurations were obtained in morin. The best ligand pose energy for fisetin (-6.15058 Kcal/mol) is found to be greater than morin (-5.86477 Kcal/mol). For the discussion, we have chosen the docked configuration of both the ligands because it posses the minimum energy. It was found that the fisetin and morin bind within H-bonding distance to tryptophan residues (Trp 62 and Trp 63) of lysozyme. The distance be present in fisetin (3$^{'}$-O to NƐ, Trp 63: 8.0 Å ,4-

C=O to NƐ, Trp 62:7.1 Å) and morin (3'-O to NƐ, Trp 63: 7.8 Å ,4-C=O to NƐ, Trp 62:4.9 Å) fig.The distance of fisetin (4'-O) to Trp 108 is found to be 5.8 Å and that for morin is (2'-O to NƐ, Trp 108) is 7.2 Å

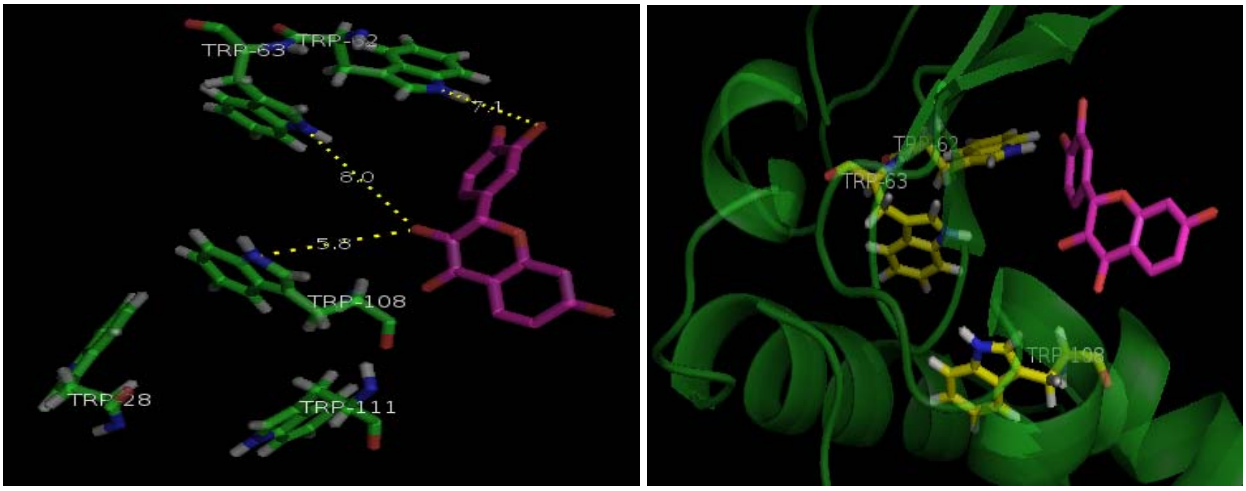


Figure 10: (a) The H-distance of a fisetin with the residue of lysozyme (b) docked poses of fisetin with lysozyme

According to the results it has also been found that ligand binds in close proximity to Trp 62 and Trp 63 of lysozyme which is the active sites for binding.

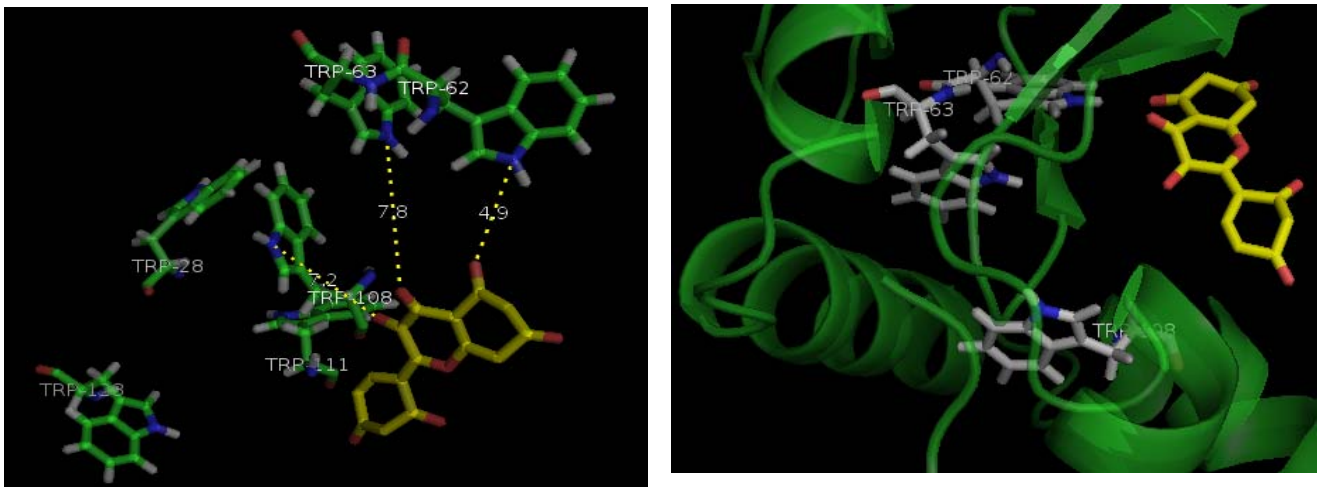


Figure 11: (a) The H-distance of a morin with the residue of lysozyme and (b) docked poses of morin with lysozyme.

The expected interaction of the polyphenols with tryptophan residues, Trp 62 and Trp 63 can also be explained on the basis of the quenching of fluorescence intensity of lysozyme by the ligands. The ΔG (free energy) and K_b (binding constant) is calculated from the molecular docking study. It is found that the binding affinity of fisetin is greater than morin as observed from the UV-vis and docking study.

Table 7: ***Binding constant and free energy from docking***

Molecule	ΔG° (kcal/mol) from docking	log K_b(docking) at 25°C	log K_b (UV-vis at 25°C)
FISETIN	-6.15	4.52	4.58
MORIN	-5.86	4.31	4.37

7. Conclusion

Polyphenols shows a broad range of biological activities that consist of antitumor, antioxidant, anticancer etc. Polyphenols used as neuroprotective agents. Considering such importance of these compounds we have focused on interaction of dietary polyphenols with lysozyme. The researches embodied in this thesis investigate the interaction of different flavonoids with lysozyme at molecular level by means of UV-vis, fluorescence anisotropy, CD and molecular docking studies. The following general points emerged from the studies reported in the thesis that described:

(a) the binding of small ligand with lysozyme

(b) the effects of pH on interaction with polyphenols

(1) Greater binding is shown in basic pH i.e pH 9.0 than acidic pH almost negligible binding.

(2) The K_b value of fisetin is found greater than morin.

(3) Binding constant is found to be order of 10^4 M^{-1}

(4) CD results show that during binding process the flavonoids are capable to increases the helical content of lysozyme (change in secondary structure)

(5) It has been found that near Trp 62 and Trp 63 ligands binds.

(6)The binding affinity of fisetin is found to be greater than morin using UV-vis and docking study.

REFERENCES

1. Papadopoulou, A., Green, R. J., Frazier, R. A.(2005) ,Interaction of flavonoids with bovine serum albumin: A fluorescence quenching study. *J. Agric. Food Chem*. 53, 158–163.
2. Rawel, H. M., Meidtner, K., Kroll, J.(2005) ,Binding of selected phenolic compounds to proteins. *J. Agric. Food Chem*. 53, 4228–4235.
3. Ahmad, E., Rabbani, G., Zaidi, N., Singh, S., Rehan, M., Khan, M.M., Rahman, S.K., Qadri, Z., Shadab, M., Ashraf, M.T., Subbarao, N., Bhat, R., Khan, R.H.,(2011) ,Stereo-selectivity of human serum albumin to enantiomeric and isoelectronic pollutants dissected by spectroscopy, calorimetry and bioinformatics, PLoS One6e26186.
4. Ahmad, E., Sen, P., Khan R.H., (2011) ,Structural stability as a probe for molecular evolution of homologous albumins studied by spectroscopy and bioinformatics,*Cell.Biochem.Biophys*.61,313–325.
5. Rabban, G.i., Ahmad ,E., Zaidi, N., Khan ,R.H., (2011) ,PH-dependent conformational transitions in conalbumin (ovotransferrin), a metalloproteinase from hen egg white,*CellBiochem.Biophys*.61,551–560.
6. Paul, B.K., Guchhait, N., (2011) ,Modulation of prototropic activity and rotational relaxation dynamics of a cationic biological photosensitizer within the motionally constrained bio-environment of a protein, *J. Phys. Chem*.B,115, 10322–10334
7. Heim, KE., Tagliaferro, AR.., Bobilya, DJ., (2002) ,Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *J Nutr Biochem* .13,572–584

8. Graf, BA., Milbury ,PE., Blumberg, JB., (2005) ,Flavonols, flavones, flavanones, and human health: epidemiological evidence. *J Med Food* .8,281–290
9. Middleton, E Jr., Kandaswami, C, Theoharides, TC., (2000) ,The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev*. 52,673– 751
10. Deaville, E. R., Green, R. J., Mueller-Harvey, I., Willoughby, I., and Frazier, R. A. (2007), Hydrolyzable tannin structures influence relative globular and random coil protein binding strengths. *Journal of Agricultural and Food Chemistry*. 55, 4554-4561.
11. Soares, S., Mateus, N., Freitas, V., (2007), Interaction of different polyphenols with bovine serum albumin (BSA) and human salivary α-amylase (HSA) by fluorescence quenching. *Journal of Agricultural and Food Chemistry*. **55**, 6726-6735.
12. Xiao, J., Cao, H., Wang, Y., Zhao, J. and Wei, X. (2009), Glycosylation of dietary flavonoids decreases the affinities for plasma protein. *Journal of Agricultural and Food Chemistry*. **57**, 6642-6648.
13. O'Connell, J.E. and Fox, P.F. (2001), Significance and applications of phenolic compounds in the production and quality of milk and dairy products: A review. *International Dairy Journal*. 11, 103-120.
14. Pignataro, B., (2008), Tomorrow's Chemistry Today, Concepts in Nanoscience, Organic Materials and Enviromental Chemistry, Chapter17: Overview of Protein-Tannin Interactions, Carvalho, E. B., Freitas, V. A. P. And Mateus, N. F. Willey-VCH Verlag GmbH & Co. KGaA, Weinheim. PP. 409-428
15. Prigent, S. V. E., Gruppen, H., Visser, A. J. W. G., Koningsveld, G. A. V., Jong G. A. H., and Voragen, A. G. J., (2003), Effects of non-covalent interactions with 5-*O*-

caffeoylquinic acid (chlorogenic acid) on the heat denaturation and solubility of globular proteins. *Journal of Agricultural and Food Chemistry*, 51,5088-5095.

16. Rawel, H. M., Meidtner, K., and Kroll, J. (2005), Binding of selected phenolic compounds to proteins. *Journal of Agricultural and Food Chemistry*.53, 4228-4235.
17. Carvalho, E., Mateus, N., Plet, B., Pianet, I., Dufourc, E. and Freitas, V. D. (2006), Influence of wine pectic polysaccharides on the interactions between condensed tannins and salivary proteins. *Journal of Agricultural and Food Chemistry*, 54, 8936-8944.
18. Ross, P. D., Subramanian, S. (1981), Thermodynamics of protein association reactions: forces contributing to stability, *Biochemistry*. 20, 3096−3102
19. Raffa, R. B. (2003), Experimental approaches to determine the thermodynamics of protein-ligand interaction, in Protein-ligand interactions: from molecular recognition to drug design, by Böhm, H. J. and Schneider, G. (Ed.), Weinheim, WILEY-VCH
20. Yang,R., Yu,L., Zeng,H., Liang,R., Chen,X., Qu,L.,The interaction of flavonoid lysozyme and relationship between molecular structure of flavonoids and their binding activity of lysozyme,*J.Fluorescence*,DOI 10.1007/s 10895-012-1082-9
21. Zhang, H.M., Chen, J.,Zhou, Q.H., Shi, Y.Q., Wang, Y.Q., (2011)Study on the interaction between cinnamic acid and lysozyme,*J.of molecular structure*.98,7-12.
22. Daojin, Li., XinXiang, Cao., Baoming, ji.,(2010), Spectrophotometeric studies on the interaction between myricetin and lysozyme in the absence or prescence of Cu^{+2} or Fe^{+3},*J.of luminescence*.130, 1893-1900.
23. Zhang, G.J., Keita, B., Miron, S., Oliveria, P.D., Nadjo, L., (2009), Biomacromolecules .9,812-817

24. Imoto, T., Forster, L.S., Rupley, J.A., Tanaka, F., (1971), Fluorescence of Lysozyme: Emissions from Tryptophan Residues 62 and 108 and Energy Migration. *Proc. Natl. Acad. Sci.* U.S.A. 69, 1151–1155 .

25. Zhang, H.X.., Huang , X., Mei, P., K.H. Li, Yan, C.N., (2006), studies on the interaction of tricyclazole with β-cyclodextrin and human serum albumin by spectroscopy. *J. Fluoresc*. 16, 287.

26. Xie, M.X., Long ,M., Liu ,Y., Qin ,C., Wang ,Y.D., (2006) ,characterization of the interaction between human serum albumin and morin. *Biochim. Biophys. Acta* ,1760-1184

27. Fleming, A. (1922), On a remarkable bacteriolytic element found in tissues and secretions, *Proc. R. Soc. Lond., B* 93, 306–317.

28. Redfield, C., Dobson, C.M. (1990), Proton NMR studies of human lysozyme: spectral assignment and comparison with hen lysozyme, *Biochemistry* .29, 7201–7214.

29. Parrot, J.L., Nicot, G. (1963), Antihistaminic action of lysozyme, *Nature* .197, 496.

30. Huang ,SL., Maiorov, V., Huang, PL., Ng A, Lee, HC., Chang, YT., Kallenbach, N., Huang, PL., Chen HC. (2005), Structural and func- tional modeling of human lysozyme reveals a unique nonapeptide, HL9, with anti-HIV activity. *Biochem* .44,4648–4655

31. Manach, C., Williamson, G., Morand, C., Scalbert, A., and Remesy C. (2005), Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *The American Journal of Clinical Nutrition*.81, 230S-242S.

32. Ignat, I., Volf, I. and Popa, V. I. (2011), Acritical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chemistry*. 126, 1821-1835.

33. Scalbert., A. and Williamson, G. (2000), Dietary intake and bioavailability of polyphenols. *The Journal of Nutrition*.130, 2073S-2085S.

34. EL Gharras., H. (2009), Polyphenols: food sources, properties and applications- a review. *International Journal of Food Science & Technology*. 44, 2512-2518.

35. Naczk, M., and Shahidi, F. (2006), Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. *Journal of Pharmaceutical and Biomedical Analysis*. 41, 1523-1542.

36. Pepys ,M.B., Hawkins, P.N., Booth, D.R. , Vigushin, D.M. , Tennent, G.A. , Soutar,N., Totty, A.K.,Nguyen, O., Blake, C.C ., Terry, C.J., et al.,(1993), Human lysozyme gene mutations cause hereditary systemic amyloidosis, *Nature* .362 , 553–557.

37. Dumoulin, M., Kumita, J.R., Dobson, C.M., (2006), Normal and aberrant biological selfassembly: insights from studies of human lysozyme and its amyloidogenic variants, *Acc. Chem. Res.* 39,603–610.

38. Dangles, O., Dufour, C., Bret, S., (1999), Flavonol-serum albumin complexation.Two-electron oxidation of flavonols and their complexes with serum albumin. *J. Chem. Soc*., Perkin Trans 2 1, 4, 737–744.

39. aki, Riihim., inio, L. H. Va., Heikura ,M. J., , Valkonen, J.M. S., Virtanen, K. H., Vuorela, V. T., (2008), Binding of phenolic compounds and their derivatives to bovine and reindeer β-lactoglobulin. *J. Agric.Food Chem*. 56, 7721–7729.

40. Soares, S., Mateus, N., De Freitas, V., (2007) , Interaction of different polyphenols with bovine serum albumin (BSA) and human salivary R-amylase (HSA) by fluorescence quenching. *J. Agric. Food Chem.* 55, 6726–6735.

41. Kim, D., Park, J.; Kim, J., Han, C., Yoon, J., Kim, N., Seo, J., Lee, C., (2006), Flavonoids as mushroom tyrosinase inhibitors: A fluorescence quenching study. *J. Agric. Food Chem*. 54, 935–941.

42. Nanda, R. K., Sarkar, N., Banerjee, R. (2007), Probing the interaction of ellagic acid with human serum albumin: A fluorescence spectroscopic study. *J. Photochem. Photobiol*. A, 192, 152–158.

43. Poncet-Legrand, C., Gautier, C., Cheynier, V., Imberty, A. (2007), Interactions between flavan-3-ols and poly(l-proline) studied by isothermal titration calorimetry: Effect of the tannin structure. *J. Agric. Food Chem*. 55, 9235–9240.

44. Wang, Y. Q., Zhang, H. M., Zhang, G. C., Tao, W. H., Fei, Z. H., Liu, Z. T. (2007), Spectroscopic studies on the interaction between silicotungstic acid and bovine serum albumin. *J. Pharm. Biomed. Anal.* 43, 1869–1875.

45. Xiao, J., Chen, X., Zhang, L., Talbot, S. G., Li, G. C., Xu, M. (2008), Investigation of the mechanism of enhanced effect of EGCG on huperzine A's inhibition of acetylcholinesterase activity in rats by multi-spectroscopic method. *J. Agric. Food Chem*.56, 910–915.

46. Geleijnse, JM., Hollman PCH (2008), Flavonoids and cardiovascular health: which compounds, what mechanism? Am J Clin Nutr. 88,12–13

47. Mabry, T. J., Markham, K. R., Thomas, M. B. (1970), The systematic identification of flavonoids. *Springer-Verlag*, New York.

48. Foti, M., Piattelli, M., Baratta, M. T., Roberto, G. (1996), Flavonoids, coumarins, and cinnamic acids as antioxidants in a micellar system. Structure-Activity Relationship, *J. Agric. Food Chem.* 44, 497–501

49. Wang, S., Xue, C., Chen, X., Liu, M., Hu, Z. (2004), Study on the quantitative relationship between the structures and electrophoretic mobilities of flavonoids in micellar electrokinetic capillary chromatography, *J. Chromatogr. A* 1033, 153–159

50. Rothwell, J. A., Day, A. J., Morgan, M. R. A. (2005), Experimental determination of octanol- water partition coefficients of quercetin and related flavonoids, *J. Agric. Food Chem.* 53, 4355−4360

51. Heim, KE., Tagliaferro, AR., Bobilya, DJ., (2002), Flavonoid antiox- idants: chemistry, metabolism and structure-activity relationships. *J Nutr Biochem* .13,572–584

52. 51) Graf, BA., Milbury, PE., Blumberg, JB., (2005), Flavonols, flavones, flavanones, and human health: epidemiological evidence. *J Med Food* .8,281–290.

53. Harborne, J. B., Williams, C. A. (2000), Advances in flavonoid research since 1992, *Phytochemistry* .55, 481–504.

54. Marcolin, E., Forgiarini, L., Rodrigues, G., Tieppo, J., Borghetti, G., Bassani, V. L., Picarda, J. N., Marroni, N. P. Quercetin decreases liver damage in mice with non-alcoholic steatohepatitis, *Basic Clin. Pharmacol. Toxicol.* DOI: 10.1111/bcpt.12049

55. Carelli, S., Hebda, D. M., Traversa, M. V., Messaggio, F., Giuliani, G., Marzani, B., Benedusi, A., Di Giulio, A. M., Gorio, A. (2012), A specific combination of zeaxanthin, spermidine and rutin prevents apoptosis in human dermal papilla cells, *Expt. Dermatol.* 21, 953−955.

56. Wang, S. W., Wang, Y. J., Su, Y. J., Zhou, W. W., Yang, S. G., Zhang, R., Zhao, M., Li, Y. N., Zhang, Z. P., Zhan, D. W., Liu, R. T. (2012), Rutin inhibits β-amyloid aggregation and cytotoxicity, attenuates oxidative stress, and decreases the production of nitric oxide and proinflammatory cytokines, *Neurotoxicology* 33, 482–490.

57. Geraets, L., Haegens, A., Brauers, K., Haydock, J. A., Vernooy, J. H. J., Wouters, E. F. M., Bast, A., Hageman, G. J. (2009), Inhibition of LPS-induced pulmonary inflammation by specific flavonoids, *Biochem. Biophys. Res. Commun.* 382, 598–603

58. Khan, N., Afaq, F., Syed, D. N., Mukhtar, H. (2008), Fisetin, a novel dietary flavonoid, causes apoptosis and cell cycle arrest in human prostate cancer LNCaP cells, *Carcinogenesis* .29, 1049–1056.

59. Lim, D. Y., Park, J. H. Y. (2009), Induction of p53 contributes to apoptosis of HCT-116 human colon cancer cells induced by the dietary compound fisetin, *Am. J. Physiol. Gastrointest. Liver Physiol.* 296, 1060–1068.

60. Pamela, M., Dargusch, R., Ehren, J. L., Okada, S., Sharma, K., Schubert, D. (2011), Fisetin lowers methylglyoxal dependent protein glycation and limits the complications of diabetes, *PLoS one* 6, e21226.

61. Ragelle, H., Crauste-Manciet, S., Seguin, J., Brossard, D., Scherman, D., Arnaud, P., Chabot, G. G. (2012), Nanoemulsion formulation of fisetin improves bioavailability and antitumor activity in mice, *Int. J. Pharm.* 427, 452–459.

62. Pongsak, P., Parichat, P. (2007), Bacteriostatic effect of flavonoids isolated from leaves of Psidium guajava on fish pathogens, *Fitoterapia.* 78, 434–436

63. Kok, L. D., Wong, Y. P., Wu, T. W., Chan, H. C., Kwok, T. T., Fung, K. P. (2000), Morin hydrate: a potential antioxidant in minimizing the free-radicals-mediated damage to cardiovascular cells by anti- tumor drugs, *Life Sci.* 67, 91–99

64. Kapoor, R., Kakkar, P. (2012), Protective role of morin, a flavonoid, against high glucose induced oxidative stress mediated apoptosis in primary rat hepatocytes, *PLoS One* 7, e41663

65. Kang, D. G., Moon, M. K., Sohn, E. J., Lee, D. H., Lee, H. S. (2004), Effects of morin on blood pressure and metabolic changes in fructose-induced hypertensive rats, *Biol. Pharm. Bull.* 27, 1779–1783.

66. Hsiang, C.Y., Wu, S. L., Ho, T.Y. (2005), Morin inhibited 12-O-tetradecanoylphorbol-13-acetate- induced hepatocellular transformation via activator protein 1 signaling pathway and cell cycle progression, *Biochem. Pharmacol.* 69, 1603–1611

67. Nelson, S. D., (1982), Metabolic activation and drug toxicity, *J. Med. Chem.*, 25, 753−765. Ni, Y., Du, S., Kokot, S. (2007), Simultaneous enzymatic kinetic determination of pesticides, carbaryl and phoxim, with the ad of chemometrics. *Anal. Chim. Acta.* 584, 19–27

68. Smolelis, AN., Hartsell SE., (1949), Factors affecting the lytic activity of lysozyme. *J. Bacteriol.* 63,665-674

69. Lakowicz, J. R. (2006), Principles of Fluorescence Spectroscopy, New York, Spinger, Third Edition.

70. Jiang, M., Xie, M.X., Zheng, D., Liu, Y., Li, X.Y., Cheng, X. (2004), Spectroscopic studies on interaction of cinnamic acid and its hydroxyl derivatives with human serum albumin, *J. Mol. Struct. 692*, 71–80.

71. Chen, Y-H., Yang, J. T., Martinez, H. M. (1972), Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersion, *Biochemistry 11*, 4120–4131.

72. W.L. DeLano, The PyMoL molecular graphics system, DeLano Scientific, San Carlos, (CA), 2004.

73. Singha Roy, A., Dinda, A.K., Dasgupta. S.:(2012), Study of the interaction between fisetin and human serum albumin: a biophysical approach. Protein Pept. Lett. 19, 604–615 .

74. Singha Roy, A., Dinda, A.K., Chaudhury, S., Dasgupta, S., (2014), Binding of antioxidant flavonol morin to the native state of bovine serum albumin: Effects of urea and metal ions on the binding. *J. Lumin*. 145, 741–751.

75. Greenfield, N., Fasman, G.D., (1969) Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry* .8, 4108–4116 .

76. Staprans, I., Watanabe, S.:(1970), Optical properties of troponin, tropomyosin, and relaxing protein of rabbit skeletal muscle. *J. Biol. Chem*. 245, 5962–5966 .

77. Singha Roy, A., Ghosh, K.S., Dasgupta, S., (2013), An investigation into the altered binding mode of green tea polyphenols with human serum albumin on complexation with copper. *J. Biomol. Struct. Dyn*. 31, 1191–1206.

78. Singha Roy, A., Tripathy, D.R., Ghosh, A.K., Dasgupta, S., (2012), An alternate mode of binding of the polyphenol quercetin with serum albumins when complexed with Cu(II). *J. Lumin.* 132, 2943–2951.