



# Inactivation of Alpha-2-Macroglobulin by Photo-Illuminated Gallic Acid

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Received: 29 April 2019 / Accepted: 2 July 2019 / Published online: 20 July 2019  
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## Abstract

Gallic acid is a naturally occurring plant polyphenol found in green tea and various fruits. Under certain conditions gallic acid exhibits pro-oxidant characteristics rather than its well known antioxidant property. In the present work, we explored the interaction of gallic acid with sheep alpha-2-macroglobulin ( $\alpha_2M$ ) in the presence of light and determined the functional alteration and conformational modifications induced in  $\alpha_2M$  structure.  $\alpha_2M$  is a highly abundant homotetrameric antiproteinase glycoprotein having diverse functions. Our result suggests  $\alpha_2M$  loses almost 54% of its proteinase inhibitory activity after 2 h incubation with gallic acid in presence of light. The inactivation of  $\alpha_2M$  was due to photodynamic generation of superoxide radical and hydrogen peroxide by gallic acid. The UV/visible absorption spectra of  $\alpha_2M$  showed increase in absorbance due to complex formation with gallic acid. Intrinsic fluorescence study shows that  $\alpha_2M$ -gallic acid interaction leads to quenching of fluorescence intensity of  $\alpha_2M$  and the mechanism of quenching is found to be static in nature. Synchronous fluorescence measurements reveal that gallic acid interaction leads to change in the microenvironment around tryptophan residues of  $\alpha_2M$ . Moreover, Fourier transform infrared spectroscopy and circular dichroism spectra suggests perturbation in secondary structure of  $\alpha_2M$ . Binding parameters were investigated by spectroscopic as well as calorimetric measurements. Negative value of enthalpy change and Gibbs free energy confirms the binding process to be exothermic and spontaneous.

**Keywords** Antioxidant · Free radicals · Gallic acid · Alpha-2-macroglobulin · Antiproteinase

## Introduction

Polyphenols belongs to a family of naturally existing antioxidants which performs numerous biological functions and makes an important component of human diet [1, 2]. Gallic acid is a polyphenol, extensively disbursed in the plant kingdom present in free form or as a component of tannins such as gallotannin [3, 4]. Gallic acid content is high in some natural foods such as strawberries, bananas, lemons, honey, different berries, pomegranate, mango, gallnuts, oak bark and apple peeling [3, 5, 6]. Gallic acid is a small triphenolic (3,4,5-trihydroxybenzoic acid) low molecular weight compound, which acts as a strong antioxidant as well as a powerful

reducing agent [7]. The presence of high electron density hydroxyl group at meta and para positions contributes towards high scavenging ability of gallic acid [7–9]. Gallic acid exhibits numerous biological properties like anti-inflammatory, anti-bacterial and anti-viral property [6, 10–13]. Gallic acid derivatives such as propyl gallate are used in phytomedicines [1]. Gallic acid inhibits dyslipidaemia, hepatosteatosis and mutagenic effects of benzidine [14, 15]. Gallic acid shows anti-tumorigenic effects against prostate [3], leukemia, oral and esophageal tumors [16, 17]. The wide range significance of gallic acid is a result of an excellent balance between its antioxidant and pro-oxidant activity [1]. Gallic acid induces death in cancer cells with higher sensitivity than normal cells [17]. The property of apoptosis induction is primarily due to its pro-oxidant behavior [9] and is fairly specific characteristic of gallic acid [18]. Pro-oxidant behaviour of gallic acid, under certain conditions, generates reactive oxygen species (ROS) and induces oxidative stress [19]. One of the derivatives of gallic acid found in green tea, epigallocatechin-3-gallate (EGCG) is very efficient pro-oxidant which reduces Cu(II) to Cu(I) leading to the formation of ROS (hydroxyl radical,

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superoxide anion and hydrogen peroxide) [20]. ROS induces oxidative damage to macromolecules (proteins, lipids and DNA) which gathers with age and progresses to various pathological conditions including neuro-degenerative disorders and cardiovascular problems [21]. It is well known that increase in ROS level by an internal or external cause brings about breaching of antiproteinase barrier often culminating in development of pathological state [22].

Alpha-2-macroglobulin ( $\alpha_2$ M) is an important component of antiproteinase barrier present in the body fluids of mammals as well as vertebrates [23].  $\alpha_2$ M is exclusive among the various plasma antiproteinase due to its capability to prohibit endopeptidases of all types and specificity [24]. Vertebrate  $\alpha_2$ M is a large protein containing identical subunits connected by disulphide bonds forming dimer which are linked by non-covalent interactions to make a tetrameric molecule [25]. Each subunit of  $\alpha_2$ M consists of a particular sequence of amino acids named the “bait region,” [24]. When cleavage occurs in the bait region by a proteinase, conformational modifications appear in  $\alpha_2$ M leading to irrevocable binding of the proteinase without blocking the active site of proteinase [24]. Bound proteinase still possesses activity for small substrates, whereas activity for large substrate is markedly diminished as proteinase is engaged inside  $\alpha_2$ M.  $\alpha_2$ M is also used in vaccines as an adjuvant and in drug delivery system for targeted delivery [26]. Various anti-cancer drugs interact covalently with  $\alpha_2$ M and alter its function [27]. Physiological oxidant such as hypochlorous acid (HOCl) is also known to alter the structure of  $\alpha_2$ M [28]. Multiple reactive sites concealed within  $\alpha_2$ M structure suggest its varied and intricate functions as a binding, carrier and targeting protein [29]. Present work is the first attempt to explore the binding of small exogenous physiologically relevant polyphenol-gallic acid with key proteinase inhibitor- $\alpha_2$ M. This study investigates pro-oxidant behavior of gallic acid in presence of light, when incubated with  $\alpha_2$ M by using free radicals scavengers and superoxide radical detection assay. The consequent loss in functional activity of  $\alpha_2$ M and conformational changes induced by gallic acid has been monitored by employing various spectroscopic techniques and by isothermal titration calorimetry for the first time. Binding parameters were also investigated by spectroscopic as well as calorimetric measurements.

## Materials and Methods

### Materials

Trypsin, nitrobluetetrazolium (NBT), bovine erythrocyte superoxide dismutase (SOD), thiourea, sodium benzoate, catalase, gallic acid, soybean trypsin inhibitor (STI) and N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) were acquired from Sigma Chemical Co. (St. Louis, MO). Chemicals required for

electrophoresis were purchased from Merck. All reagents were analytical grade and of highest purity commercially available.

## Methods

### Purifications of $\alpha_2$ M

Fresh sheep blood was collected in container having 1/10 volume of acid citrate dextrose and 1 mM PMSF/STI (50 mg/l of blood) at slaughter house (immediately after slaughtering). Isolation and purification of  $\alpha_2$ M from sheep blood was performed by the procedure of Rehman et al., [30, 31]. Sheep  $\alpha_2$ M was purified by ammonium sulphate fractionation and gel filtration chromatography. The ammonium sulphate fraction which gets precipitated between 20% and 40% saturation was dialysed against 50 mM sodium phosphate buffer pH 7.4. Afterwards it is chromatographed on sephacryl-S-300 HR column in phosphate buffer (pH 7.4). The protein activity was then determined by activity assay and the fractions having inhibitory activity against trypsin were concentrated. 10  $\mu$ g of protein sample was loaded onto wells and run on 5% non-denaturing PAGE [32] using tris-glycine buffer (pH 8.3). Staining of gel was performed with 0.15% CBBR-250 for 20 min then washed carefully and destained. Purified  $\alpha_2$ M gave a single clear band in gel suggesting electrophoretic homogeneity.  $\alpha_2$ M was stored at 4 °C (for short term) and was checked before use by PAGE to assure  $\alpha_2$ M purity [33].

### Reaction of Gallic Acid with $\alpha_2$ M

Gallic acid stock solution (1 mM) was freshly made in sodium phosphate buffer, pH 7.4 and further diluted to obtain 10–50  $\mu$ M gallic acid. Purified sheep  $\alpha_2$ M (10  $\mu$ M) was treated with increasing concentrations of gallic acid (10–50  $\mu$ M) in total volume of 1 ml at 25 °C under light of 400 flux from a fluorescent lamp for 2 h. On completion of reaction, inhibitory activity of  $\alpha_2$ M was evaluated and other spectroscopic techniques were carried out to determine alteration in structure and conformations of  $\alpha_2$ M. The experiments were carried out in triplicates before reporting the final result.

### Assay of $\alpha_2$ M Antiproteinase Activity

Activity of  $\alpha_2$ M was quantitated by the capability of  $\alpha_2$ M to shield the amidolytic activity of trypsin from an excess of soybean trypsin inhibitor (STI) [34]. Briefly,  $\alpha_2$ M alone and  $\alpha_2$ M prior treated with gallic acid (10–50  $\mu$ M) for 2 h was incubated with trypsin (100  $\mu$ l) for 15 min at 37 °C. STI (100  $\mu$ l) was then added and allowed to react for another 15 min. BAPNA- the synthetic substrate of trypsin was now added (2 ml) to this mixture and incubated for 30 min. The activity of native  $\alpha_2$ M was taken as reference. Protein activity

was also assayed by varying time of incubation of reaction mixture for 1–4 h. In some experiments free radical scavengers such as superoxide dismutase, catalase and thiourea were added to determine which radical predominates in destructing activity of treated  $\alpha_2M$  [35].

### Superoxide Radical Assay

The superoxide radicals generated in the reaction mixture were detected by their ability to reduce p-nitrobluetetrazolium (NBT) to a formazan [36]. The experiment was performed by adding 50 mM sodium phosphate (pH 7.4), 33 mM NBT, 0.1 mM EDTA and 0.06% triton x-100 in final volume of 3 ml [36]. Reaction was initiated by incubating gallic acid (50  $\mu M$ ) under light from fluorescent lamp and measuring the absorbance against a blank which did not contain gallic acid. In this method, the superoxide anion, if generated, reduces the yellow colored dye NBT and forms blue formazan, measured at 560 nm [7]. In control, SOD was added prior to the addition of gallic acid to confirm the formation of superoxide anion.

### UV/Visible Absorption Spectroscopy

UV/visible spectroscopy is based on degree of absorption of UV-visible light by the sample. The UV-Visible spectra measurements were performed on Perkin-Elmer Lambda 25 double beam spectrophotometer. The absorbance was recorded over the wavelength range of 250–350 nm in a cuvette of path length of 1 cm.

### Intrinsic Fluorescence Measurements

The fluorescence spectroscopy was performed on a Shimadzu RF-5301 spectrophotometer (Tokyo, Japan) using a quartz cuvette of cell length 10 mm. Intrinsic fluorescence was measured by exciting the protein solution at a wavelength of 280 nm and collecting the emission spectra at a wavelength range of 300–400 nm. Slit width for excitation and emission was set at 5 nm. The emission spectrum of gallic acid alone was also taken to preclude the probability of fluorescence by itself.

The fluorescence data were analyzed by the linear Stern-Volmer eq. (1) [37]:  $F_0/F = K_{sv}[Q] + 1 = k_q\tau_0[Q] + 1$ ; where  $F_0$  and  $F$  are the fluorescence intensities in the absence and presence of gallic acid,  $K_{sv}$  is the Stern–Volmer quenching constant,  $[Q]$  is the concentration of quencher,  $k_q$  is the bimolecular rate constant of the quenching reaction and  $\tau_0$  is the average integral fluorescence life time of tryptophan ( $10^{-8}$  s) [38, 39]. Furthermore, the binding constant ( $K_b$ ) and the stoichiometry ( $n$ ) of binding were obtained by modified Stern-Volmer eq. (2) [40]:  $\log[(F_0 - F)/F] = \log K_b + n \log [Q]$ ; where  $[Q]$  is the concentration of gallic acid. The change in free

energy ( $\Delta G$ ) was calculated from Gibbs-Helmholtz eq. (3) [41]:  $\Delta G = RT \ln K_b$ ; where  $R$  ( $1.987 \text{ cal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ ) is gas constant and  $T$  is absolute temperature (298 K).

### Synchronous Fluorescence Measurements

The synchronous fluorescence spectra were analyzed by scanning the excitation and emission monochromator together [42]. When the difference between the excitation and emission wavelength ( $\Delta\lambda$ ) was 60 nm, synchronous fluorescence explains about the changes in microenvironment in proximity of tryptophan. When the difference was 15 nm, it describes about the possible variations in microenvironment of tyrosine [43]. Synchronous fluorescence spectra were recorded over the wavelength of 280–400 nm.

### Circular Dichroism Measurements

CD spectroscopy was accomplished on a JASCO CD J-815 spectropolarimeter at 25 °C. Far-UV CD spectra were recorded in a quartz cuvette of 1 mm path length in wavelength range 190–250 nm. CD spectral analysis was carried out to elucidate alterations in secondary structure of  $\alpha_2M$  due to gallic acid. Three scans were acquired with a scanning speed of 500 nm/min.

### Fourier Transform Infrared Spectrometry

FTIR spectrometry was performed on Perkin-Elmer Spectrum Two spectrometer over the wave number ranging from 1600 to 1700  $\text{cm}^{-1}$  to record the characteristic amide I band. The absorption peak in an infrared spectrum correlates to the frequencies of vibration of the bonds in between atoms. FTIR spectra were carried out to detect alteration in secondary structure of protein and the curve-fitted result of the amide I band.

### Isothermal Titration Calorimetry

The changes in thermodynamic parameters were achieved on a VPC-ITC Micro Cal (GE Healthcare, USA). ITC reveals the value of entropy change, enthalpy change, Gibbs free energy change, number of binding sites and binding affinity of  $\alpha_2M$ -gallic acid interaction. Initially, the samples were degassed by a thermovac unit equipped with the instrument [44]. The sample cell was loaded with  $\alpha_2M$  (1  $\mu M$ ), reference cell was brimmed with 50 mM sodium phosphate buffer and the syringe was filled up with gallic acid (20  $\mu M$ ). Subsequent titrations were carried out while stirring the sample solution repeatedly at 307 rpm [44]. The calorimetric data was recorded using the MicroCal Origin software supplied with the instrument. Heat of dilution for gallic acid were determined by control experiments and subtracted from the integrated data before curve fitting.

## Statistical Analysis

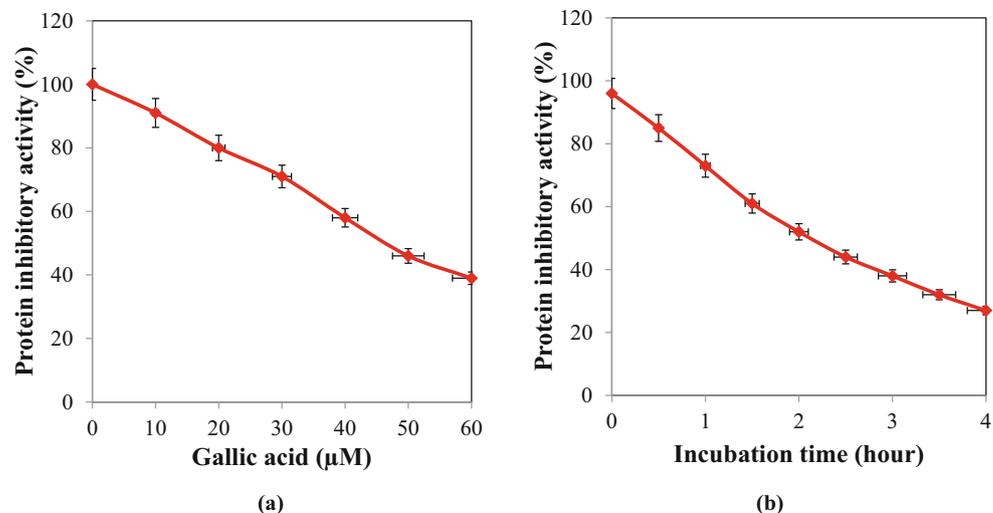
The data in results are expressed as mean  $\pm$  standard deviation ( $\pm$ SD) with  $n = 3$  representing the number of independent experiments.

## Results

### Effect of Gallic Acid on Antiproteinase Activity of $\alpha_2$ M

To quantitate the effect of increasing concentration of gallic acid (10–50  $\mu$ M) on  $\alpha_2$ M functions, antiproteolytic potential of  $\alpha_2$ M was evaluated by the amidase assay. As demonstrated in Fig. 1(a), native  $\alpha_2$ M without gallic acid exhibit maximum activity and was considered as reference (100%). On increasing gallic acid concentration from 10  $\mu$ M to 50  $\mu$ M, antiproteinase activity of  $\alpha_2$ M show dose dependent decrease, i.e. the capability to bind with proteinase decreases. At high concentration (50  $\mu$ M) of gallic acid, only 46% residual protein activity was left. This data illustrates that  $\alpha_2$ M exposure to photo-illuminated gallic acid compromises significant amount of proteinase inhibitory activity. Activity of photo-illuminated  $\alpha_2$ M was also determined to ensure that the process itself does not alter the protein activity and was found to be the same ( $\sim$ 98%) as that of native protein. We also investigated effect of incubation of gallic acid (50  $\mu$ M) for increasing time period (1–4 h). As expected  $\alpha_2$ M antiproteinase function was found to decrease with increase in incubation with gallic acid in light. Figure 1(b) depicts that  $\alpha_2$ M losses its antiproteinase activity in a time dependent manner. After 2 h incubation, about 50% residual activity remained in  $\alpha_2$ M. Control experiments where  $\alpha_2$ M was exposed to gallic acid (50  $\mu$ M) in complete darkness exhibited no significant loss (less than 2%) in proteinase inhibitory function.

**Fig. 1** a Effect of gallic acid (10–50  $\mu$ M) on proteinase inhibitory activity of  $\alpha_2$ M (10  $\mu$ M) b Time (1–4 h) dependent interaction of gallic acid (50  $\mu$ M) with  $\alpha_2$ M (10  $\mu$ M)

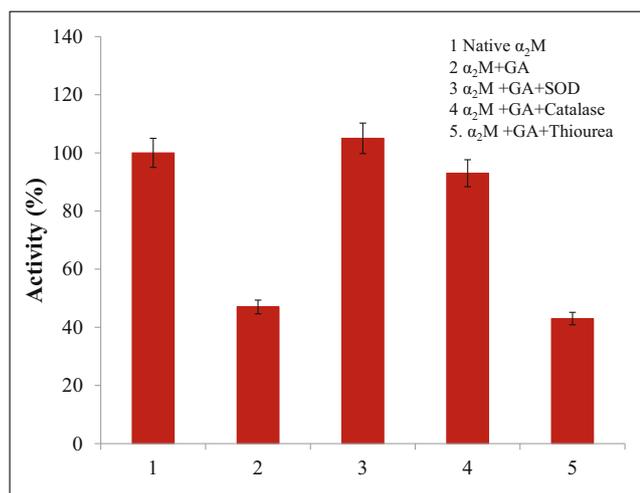


### Effect of ROS Scavengers on Activity of Gallic Acid Treated $\alpha_2$ M

Gallic acid is known to show pro-oxidant property and generate ROS such as hydroxyl radical, superoxide radicals and hydrogen peroxide [19, 45]. The active participation of these ROS in  $\alpha_2$ M inactivation was investigated via experiments involving various antioxidant enzymes and radical scavengers. Gallic acid-induced  $\alpha_2$ M inactivation was inhibited by several radical scavengers. Superoxide dismutase (SOD), catalase are the major intracellular antioxidant defenses of mammalian cells. SOD eliminates superoxide radical, catalase eliminates hydrogen peroxide and thiourea scavenges hydroxyl radicals [35]. It was found that addition of SOD (0.1 mg/ml) or catalase (0.1 mg/ml) preserved  $\alpha_2$ M functional activity to its near original state. This suggests possible involvement of superoxide radical and hydrogen peroxide in  $\alpha_2$ M inactivation. The lack of protection by hydroxyl radical scavenger thiourea (50 mM) hints that damaging species is not hydroxyl radical (Fig. 2). The above data suggests that superoxide radicals and hydrogen peroxide generated by photo-illuminated gallic acid (50  $\mu$ M) oxidatively modify the antiproteinase and compromises its antiproteinase potential. The effect of SOD and catalase alone on  $\alpha_2$ M activity was also checked and was found negligible.

### Superoxide Radical Assay

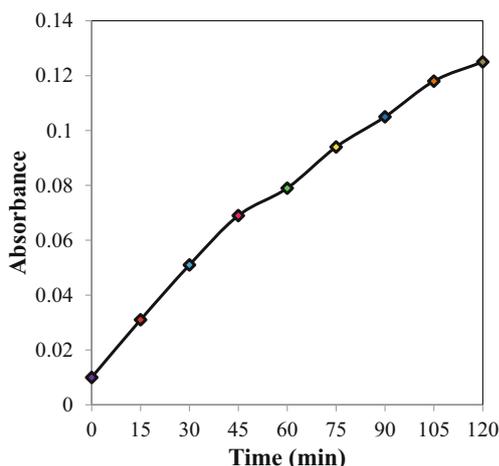
Superoxide anions generated on photo-illumination of gallic acid (50  $\mu$ M) were evaluated by NBT reduction assay [7, 36]. Our result suggests generation of superoxide radicals increases with increase in time of photo-illumination (15–120 min) of gallic acid as shown in Fig. 3. Control experiments containing gallic acid in complete darkness does not lead to any generation of superoxide radical.



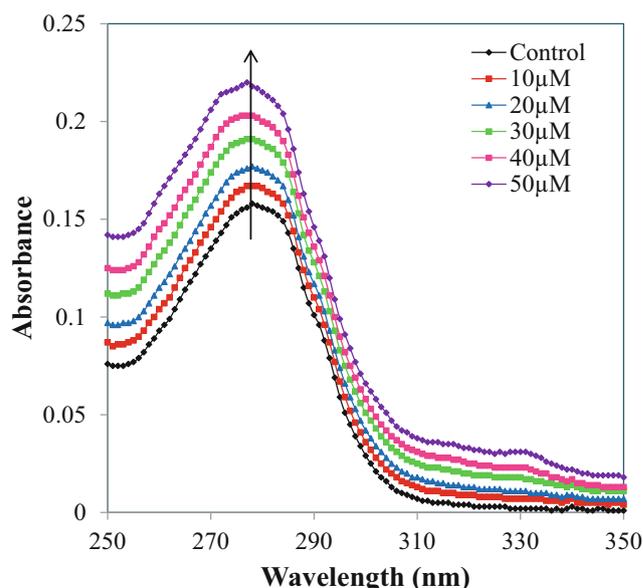
**Fig. 2** Effect of free radical scavengers [SOD (0.1 mg/ml), catalase (0.1 mg/ml) and thiourea (50 mM)] on percent residual activity of  $\alpha_2M$  (10  $\mu M$ ) after treatment with gallic acid (50  $\mu M$ ) for 2 h

**UV-Visible Absorption Studies**

UV/Visible spectroscopy is used to probe the structural transition and to affirm the complex formation between protein and metabolite [46]. Aromatic amino acids are responsible for the distinctive peak of  $\alpha_2M$  spectra at 280 nm which is consistent with previous report [28]. On addition of increasing concentration of gallic acid (10–50  $\mu M$ ), absorbance increases progressively i.e., hyperchromicity was observed. Figure 4 shows the absorption spectra of  $\alpha_2M$  alone and with increasing concentration of gallic acid. Increase in absorption intensity suggests complex formation between ligand and  $\alpha_2M$  [43]. Results indicate that gallic acid interacted with  $\alpha_2M$ .



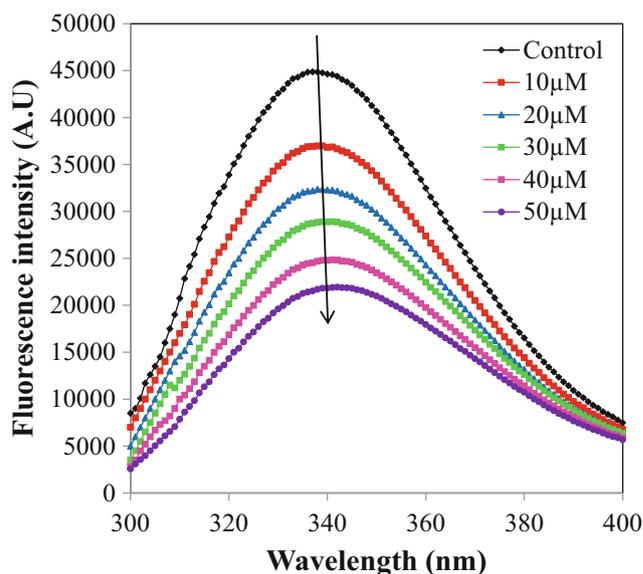
**Fig. 3** Detection of superoxide radical generated by photo-illumination of gallic acid (50  $\mu M$ ). The absorbance was recorded at 560 nm. Experiments were performed in triplicates and expressed as mean  $\pm$  S.D.



**Fig. 4** Absorption spectra of native and gallic acid (10–50  $\mu M$ ) treated  $\alpha_2M$  (10  $\mu M$ )

**Mechanism of Fluorescence Quenching of  $\alpha_2M$  by Gallic Acid**

Intrinsic fluorescence quenching studies were performed to acquire information on gallic acid induced structural change in  $\alpha_2M$ . Fluorescence shown by proteins is because of the intrinsic fluorophore (tryptophan and tyrosine residues) present in them [42]. Figure 5 illustrates that  $\alpha_2M$  show a distinctive emission spectrum falling in the wavelength range of 300–400 nm with maximum fluorescence intensity at 340 nm. Progressive treatment with gallic acid from 10



**Fig. 5** Intrinsic fluorescence spectra of native  $\alpha_2M$  (10  $\mu M$ ) and gallic acid (10–50  $\mu M$ ) treated  $\alpha_2M$

50  $\mu\text{M}$  steadily quenches the fluorescence intensity of  $\alpha_2\text{M}$ . This decline in fluorescence intensity of  $\alpha_2\text{M}$  on addition of gallic acid is an indication of protein-ligand interaction [44, 46]. Emission spectrum of gallic acid alone was also taken to preclude the probability of fluorescence by ligand and was negligible. Intrinsic fluorescence probes the interaction between protein and ligand on the basis of quenching [46]. Quenching is defined as decline in fluorescence intensity due to some molecular rearrangements, energy transfer processes, static quenching and collisional quenching [40, 46]. Fluorescence quenching can be either dynamic or static or both [40, 47]. Dynamic quenching is due to collisional encounter between the fluorophore and the ligand whereas static quenching is the result of ground state complex formation between the fluorophore and the ligand. This quenching in fluorescence intensity upon addition of gallic acid was analyzed according to the Stern–Volmer eq. (1). A linear dependence between  $F_0/F$  and molar concentration of gallic acid (1:1) (Fig. 6a) confirms the occurrence of single quenching mechanism which can either be static or dynamic. Furthermore, to determine the type of quenching occurring in gallic acid- $\alpha_2\text{M}$  system, Stern–Volmer quenching constant ( $K_{sv}$ ) and bimolecular quenching constant ( $K_q$ ) were evaluated using Stern–Volmer eq. (1). The Stern–Volmer quenching constant was found to be  $1.99 \times 10^4 \text{ M}^{-1}$ . The bimolecular quenching constant ( $k_q$ ) for gallic acid- $\alpha_2\text{M}$  interaction was found to be  $1.99 \times 10^{12} \text{ M}^{-1} \text{ s}^{-1}$  which is 100 times higher than the maximum scatter collision quenching constant of various quenchers with biopolymers ( $2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ ) [48]. This indicates that the probable quenching mechanism involves static quenching, and is not initiated by dynamic diffusion instead occurs by formation of a complex between gallic acid and  $\alpha_2\text{M}$  [49]. The binding constant and the binding stoichiometry were calculated from modified Stern–Volmer eq. (2). Plot of  $\log[(F_0/F)-1]$  vs.  $\log[\text{gallic acid}]$  gives a straight line as shown in Fig. 6b. Binding parameters obtained are tabulated in Table 1. The value of binding constant ( $K_b$ )

**Table 1** Binding parameters of  $\alpha_2\text{M}$ -gallic acid interaction obtained from fluorescence experiment at  $T = 298 \text{ K}$

Parameters	Value
Stern–Volmer constant ( $K_{sv}$ )	$1.99 \times 10^4 \text{ M}^{-1}$
Quenching constant ( $k_q$ )	$1.99 \times 10^{12} \text{ M}^{-1} \text{ s}^{-1}$
Binding constant ( $K_b$ )	$2.87 \times 10^4 \text{ M}^{-1}$
Stoichiometry (n)	1.01
Gibbs energy change ( $\Delta G$ )	$-6.32 \text{ kcalmol}^{-1}$

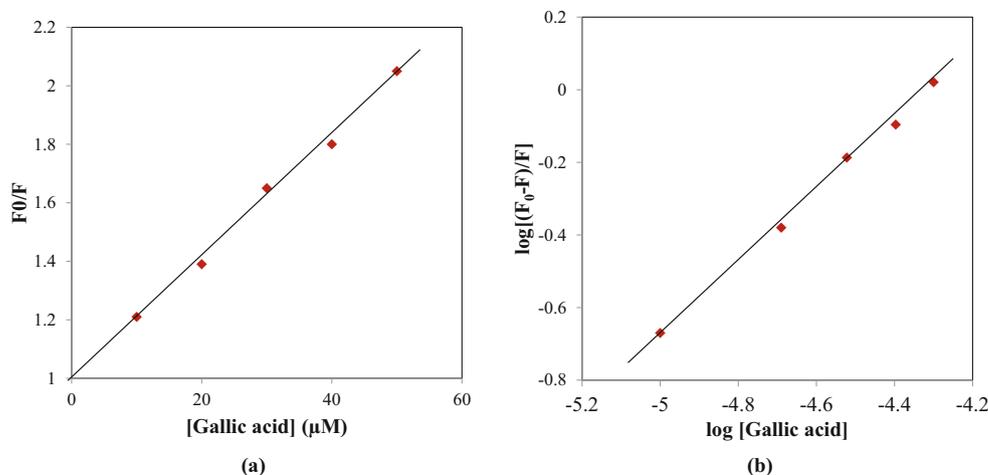
\*R<sup>2</sup> for all the values ranges from 0.98 to 0.99

was found to be  $2.87 \times 10^4$  which suggests moderate binding of gallic acid to  $\alpha_2\text{M}$ . The obtained value of n indicates the stoichiometry of gallic acid interaction with is with one binding site of protein. The change in free energy ( $\Delta G$ ), calculated from Gibbs–Helmholtz eq. (3) gives a negative value of free energy change ( $\Delta G = -6.32 \text{ kcalmol}^{-1}$ ) which represents a spontaneous occurrence of the reaction [50].

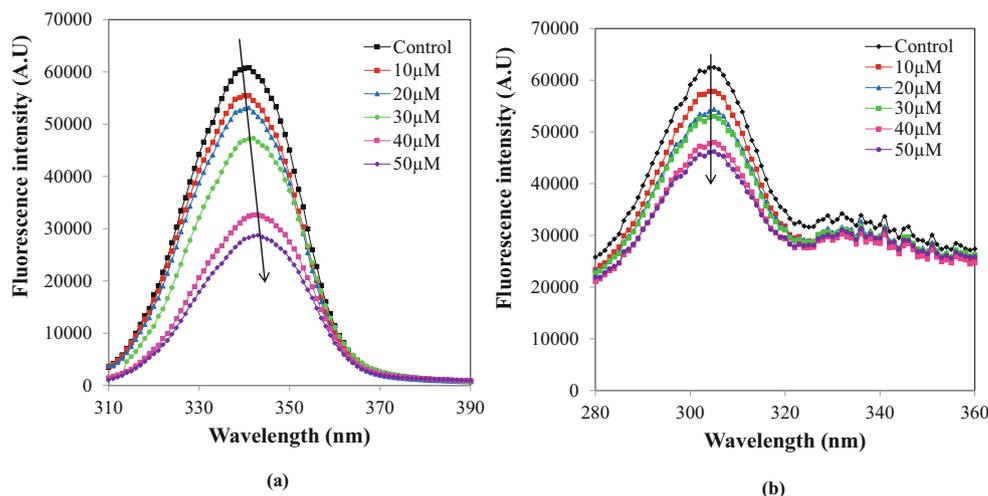
### Synchronous Fluorescence Measurements

Alteration in microenvironment around fluorophores (specifically tyrosine and tryptophan) present in protein was observed through synchronous fluorescence [41]. Figure 7 displays synchronous fluorescence profile of  $\alpha_2\text{M}$  with varying gallic acid concentration. Our results suggest as we titrate  $\alpha_2\text{M}$  with gallic acid, a subtle red shift in the peak (up to 4 nm) was noticed (when  $\Delta\lambda = 60 \text{ nm}$ ) which signifies slight change in the microenvironment around tryptophan residue [51]. However addition of gallic acid did not cause any shift (when  $\Delta\lambda = 15 \text{ nm}$ ) suggesting gallic acid interaction with  $\alpha_2\text{M}$  did not bring about change in the environment around tyrosine residues. However, fluorescence intensity decreases in both systems. Our data suggests that interaction of gallic acid with  $\alpha_2\text{M}$  drive the tryptophan residue towards hydrophilic surroundings [41, 52]. Hence, it was concluded that tryptophan plays an

**Fig. 6** a Stern–Volmer and b modified Stern–Volmer plot of fluorescence quenching of  $\alpha_2\text{M}$  by gallic acid at  $25^\circ\text{C}$



**Fig. 7** Synchronous fluorescence spectra of  $\alpha_2M$  (10  $\mu M$ ) in absence and presence of different concentration of gallic acid (10–50  $\mu M$ ). The difference between excitation and emission wavelength ( $\Delta\lambda$ ) was (a) 60 nm and (b) 15 nm



important role during fluorescence quenching of  $\alpha_2M$  and are relocated to more polar surroundings [52].

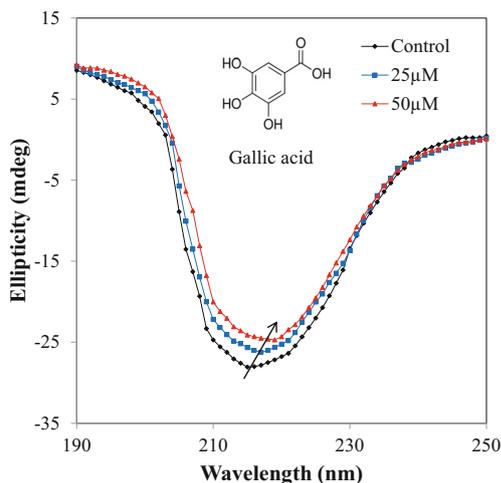
**CD Measurements**

Any kind of variation in protein secondary structure can be resolved through far UV CD spectra measurements [46]. CD spectrum of native sheep  $\alpha_2M$  shows a negative peak at 216 nm [31]. CD spectral profile of  $\alpha_2M$  in presence of two different concentrations of gallic acid viz.; 25  $\mu M$  and 50  $\mu M$  were shown in Fig. 8. Upon interaction with 25  $\mu M$  gallic acid, the negative peak of  $\alpha_2M$  was slightly shifted to 217 nm as well as a decrease in negative ellipticity was observed. On further increasing the concentration of gallic acid to 50  $\mu M$ , negative peak was shifted to 219 nm. Our observations of shifting of the peak on interaction with gallic acid points to that fact that gallic acid increase the alpha helical

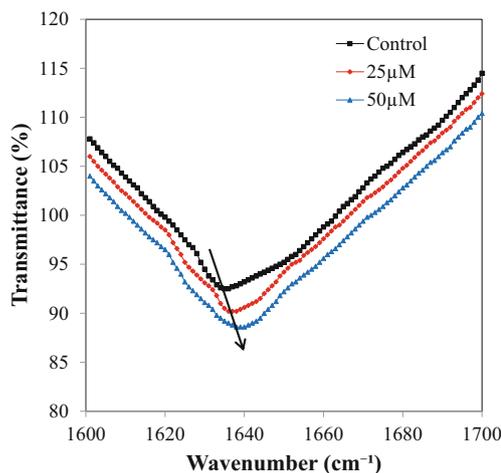
structure of  $\alpha_2M$ . However, no alteration in the shape of peak signifies that  $\alpha_2M$  is predominantly beta helix [31] in nature even after binding with gallic acid. This data affirms that alteration in  $\alpha_2M$  secondary structure arises on interaction with gallic acid. Experiments were performed in duplicate and each spectrum was average of two scans.

**FTIR Measurements**

FTIR spectroscopy was performed to gain additional information about protein secondary structure and possible structural change. Variation in secondary structure was analyzed by observing amide I band. The amide I band is the major band in the IR spectrum which is most susceptible spectral region in protein secondary structure [52]. Protein displays amide I band in 1600–1700  $cm^{-1}$  wave number range which include C=O stretching vibration of the amide group and a minor



**Fig. 8** Far-UV CD spectra of  $\alpha_2M$  alone and after treatment with gallic acid. Purified sheep  $\alpha_2M$  (10  $\mu M$ ) was incubated with 25  $\mu M$  and 50  $\mu M$  of gallic acid in sodium phosphate buffer (pH 7.4)



**Fig. 9** FTIR spectra of  $\alpha_2M$  alone and after treatment with gallic acid. Purified sheep  $\alpha_2M$  (10  $\mu M$ ) was incubated with 25  $\mu M$  and 50  $\mu M$  of gallic acid in sodium phosphate buffer (pH 7.4)

contributions from C-N stretching vibration [53]. The FTIR spectra of  $\alpha_2\text{M}$  shows amide I band at  $1635\text{ cm}^{-1}$  (Fig. 9) which endorse the predominant  $\beta$ -structure of  $\alpha_2\text{M}$  [54]. On treatment with  $25\text{ }\mu\text{M}$  of gallic acid, the amide I band deviated to  $1639\text{ cm}^{-1}$  indicating structural change in native  $\alpha_2\text{M}$ . Additionally, when  $\alpha_2\text{M}$  was treated with  $50\text{ }\mu\text{M}$  of gallic acid, amide I band showed additional shift to  $1641\text{ cm}^{-1}$ . Results indicate that gallic acid interact with C-N groups in the protein polypeptides and cause rearrangement of the polypeptide carbonyl hydrogen bonding network [54]. The observed decrease in transmittance of the amide I band in the presence of gallic acid is due to the reduction of protein beta helical content leading to change in  $\alpha_2\text{M}$  secondary structure corroborating our CD results.

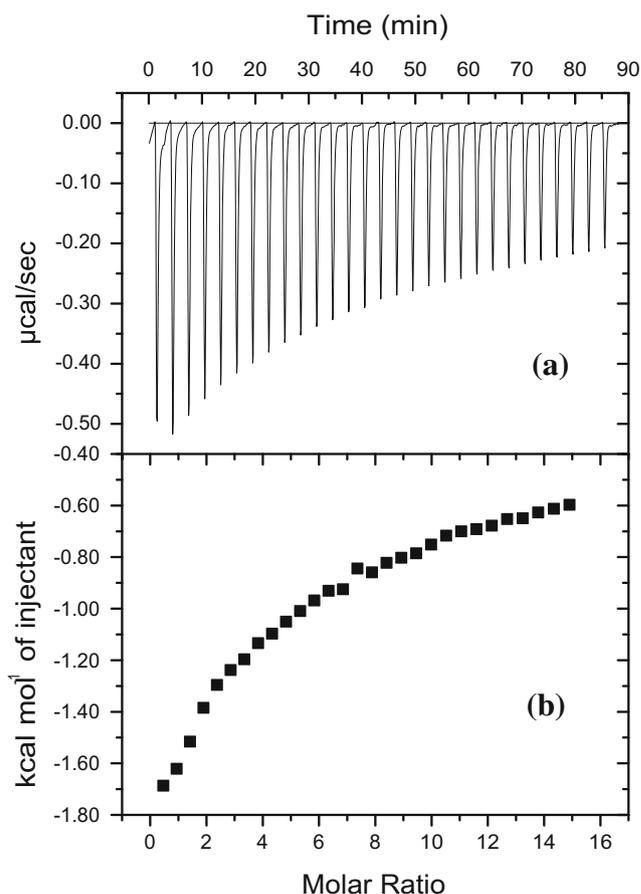
### Isothermal Titration Calorimetric (ITC) Analysis

ITC was employed to determine the magnitude of binding forces involved during protein-gallic acid interaction as well as to gain knowledge about the spontaneity of the reaction [54]. ITC interprets change in entropy ( $\Delta S$ ), change in enthalpy ( $\Delta H$ ), Gibbs free energy ( $\Delta G$ ), number of binding sites ( $N$ ) and binding affinity constant ( $K$ ) (Table 2). The calorimetric data was subtracted for the corresponding blank measurements. As shown in Fig. 10(a), every peak of binding isotherm symbolizes a single round of injection of gallic acid into the  $\alpha_2\text{M}$  solution [31]. Figure 10(b) shows the profile of heat liberated per injection as a function of molar ratio of gallic acid into  $\alpha_2\text{M}$ . The thermodynamic values ( $K_b$  and  $\Delta H$ ) were directly obtained after the best fitting for the integrated heats using single set of binding model with lowest chi square value. The 1:1 binding stoichiometry of gallic acid to  $\alpha_2\text{M}$  with a binding constant of  $2.87 \times 10^4\text{ M}^{-1}$  indicates moderate and specific interaction [37]. The Gibbs free energy and entropy changes were calculated from equation:  $\Delta G = \Delta H - T\Delta S$ . The negative enthalpy value ( $\Delta H = -2.61\text{ kcalmol}^{-1}$ ) and positive entropy value ( $T\Delta S = 4.3\text{ kcalmol}^{-1}$ ) accounts for negative value of free energy ( $\Delta G = -6.91\text{ kcalmol}^{-1}$ ) which suggests gallic acid- $\alpha_2\text{M}$  interaction is more feasible and hence

**Table 2** Thermodynamic parameters for the interaction of gallic acid with  $\alpha_2\text{M}$  in 50 mM sodium phosphate buffer (pH 7.4) obtained by ITC, at  $37\text{ }^\circ\text{C}$

Parameters	Value
Stoichiometry (n)	1.04
Binding constant ( $K_b$ )	$2.72 \times 10^4\text{ M}^{-1}$
Enthalpy change ( $\Delta H$ )	$-2.61\text{ kcalmol}^{-1}$
Entropy change ( $T\Delta S$ )	$4.3\text{ kcalmol}^{-1}$
Gibbs free energy change ( $\Delta G$ )	$-6.91\text{ kcalmol}^{-1}$

The calorimetric data was subtracted for the corresponding blank measurements



**Fig. 10** Thermogram (a) and binding isotherm (b) for the titration of gallic acid with  $\alpha_2\text{M}$  at  $37\text{ }^\circ\text{C}$ . Sample cell contains  $\alpha_2\text{M}$  ( $1\text{ }\mu\text{M}$ ), syringe contains gallic acid ( $20\text{ }\mu\text{M}$ ) and reference cell contains 50 mM sodium phosphate buffer (pH 7.4)

spontaneous. Our results of ITC are consistent with that of our fluorescence quenching results.

The negative enthalpy value and positive entropy value accounts for negative value of free energy which suggests gallic acid- $\alpha_2\text{M}$  interaction is more feasible and hence spontaneous [53, 54]. The negative enthalpy change indicates the reaction is exothermic and possibly involves hydrogen bonding forces in gallic acid-  $\alpha_2\text{M}$  interaction [55, 56]. The positive entropy value was usual of hydrophobic effect and signifies increased hydrophobic forces in the reaction system [57].

### Discussion

Gallic acid has applications in food processing, cosmetics and in packing materials of edible items to protect from spoilage and rancidity caused by lipid peroxidation [19]. It displays the massive antioxidant capability among the diverse range of polyphenols [1, 58]. Antioxidant property of gallic acid was considerably influenced by transition metals as a consequence of which it can either quench or stimulate free radical

generation via metal chelation [1, 59]. Various in vitro investigations have shown that gallic acid stimulates hydroxyl radical generation in presence of Fe(II) or Fe(III), while it functions as hydroxyl radical scavenger in absence of Fe(II) or Fe(III) [19, 59]. Pro-oxidative response of gallic acid is mediated primarily by the production of ROS such as hydroxyl radicals, hydrogen peroxide and superoxide anion [60, 61]. The superoxide anion generated by gallic acid can dismutate to hydrogen peroxide which could generate potent hydroxyl radical via iron-catalyzed Haber-Weiss reaction [62]. Gallic acid is known to have cytotoxic effect on isolated hepatocytes [63]. Pro-oxidative property of gallic acid induces cell death in human glioblastoma cells [64] and HL-60 cell line [65].

ROS is kept in fine balance in the body. An increase in ROS level by any internal or external factor can bring about damage to cellular and extra milieu including that to proteins, proteinases and anti proteinases [22]. Damage to any member of antiproteinase barrier will shift the equilibrium between proteinase and antiproteinases in favour of proteinase resulting in development of clinical pathologies [22].  $\alpha_2\text{M}$  represents an important component of antiproteinase barrier and is considered as “reserve force” of antiproteinase army. The major role of antiproteinase mainly  $\alpha_2\text{M}$  is to rapidly clear the excess proteinase released during tissue injury, infections etc. protecting the body from uncontrolled proteolytic activity which could lead to the development significant patho-physiological conditions [22, 27]. The correlation between oxidative stress and the cellular proteinase-antiproteinase balance has been a major component in the development of several pathologies [66].

Even though significant amount of studies have reported about the pro-oxidant and antioxidant activities of gallic acid [19], knowledge of interaction between  $\alpha_2\text{M}$  and gallic is still not forthcoming.  $\alpha_2\text{M}$  is known to bind, carry metal ions [67], distribute and clear variety of cytokines [26, 68, 69] and hormones [68]. Previous studies have shown that binding of small hydrophobic molecules and cytokines do not affect the conformation of the  $\alpha_2\text{M}$  or affects its inhibitory potential [33, 70]. Our study attempts to explore the interaction of a small polyphenol-gallic acid with  $\alpha_2\text{M}$  and characterize the nature of interaction. We also report functional inactivation of antiproteinase- $\alpha_2\text{M}$  by photo-illuminated gallic for the first time. On interaction with known antioxidant gallic acid under visible light,  $\alpha_2\text{M}$  was incapable to entirely protect the trapped trypsin from inhibition by soybean trypsin inhibitor and lost more than 50% of its antiproteolytic potential within short period (2 h at 50  $\mu\text{M}$  gallic acid concentration). The plausible mechanism of  $\alpha_2\text{M}$  inactivation by gallic acid was found to be the formation of superoxide radical and hydrogen peroxide. These ROS generated by photo-illuminated gallic acid could either cause possible oxidation of critical amino acid residues of  $\alpha_2\text{M}$  so that proteinases no longer bind to it or may induce subtle structural changes in  $\alpha_2\text{M}$  which may affect its trap

closing ability and hence causing loss of functional activity. Superoxide anion are known to inactivate  $\alpha_2\text{M}$  [71] and their generation is unequivocally demonstrated in our studies.

Multi-spectroscopic techniques confirm the structural and conformational changes induced in  $\alpha_2\text{M}$  on interaction with gallic acid. An increase in absorption intensity of  $\alpha_2\text{M}$  on treatment with gallic acid (10-50  $\mu\text{M}$ ) in UV/Visible absorption spectra is suggestive of complex formation between the protein and ligand [46]. Intrinsic fluorescence studies exhibit progressive spectral quenching of  $\alpha_2\text{M}$  in a concentration dependent manner of gallic acid indicating structural modifications in  $\alpha_2\text{M}$  [44, 46]. The mechanism of quenching is found to be static in nature which occurs due to ground state complex formation. Synchronous fluorescence spectra suggest microenvironment change around tryptophan residues and decrease in hydrophobicity around them [52, 54]. Assessment by FTIR and CD affirms the perturbation in the secondary structure of  $\alpha_2\text{M}$  after exposure with gallic acid. The binding parameters obtained by fluorescence quenching studies are consistent with that determined by ITC. The negative value of  $\Delta H$ , positive value of  $\Delta S$  and moderate value of binding affinity constant ( $\sim 10^4 \text{ M}^{-1}$ ) confirms the binding interaction of gallic acid with  $\alpha_2\text{M}$  to be exothermic and spontaneous. Small molecules with hydroxyl and carboxyl groups are known to interact with proteins carboxyl and NH group and render conformational change in protein at low concentration [70]. Hydrogen bonding occurs between the phenolic hydroxyl group of gallic acid and the amide group of  $\alpha_2\text{M}$ . This was due to the fact that phenol can be absorbed on the protein surface and can interact with protein in either reversible or irreversible manner leading to conformational alterations [72]. Gallic acid did induce significant loss in antiproteinase activity and caused subtle but clear changes in conformation of key antiproteinase of serum. Any change in conformational status of  $\alpha_2\text{M}$  is associated with altered binding of cytokines [26] and changed functional status [28] which has multidimensional effects.

## Conclusion

This study is the first attempt to explore the interaction of photo-illuminated gallic acid with  $\alpha_2\text{M}$  and its effect on functional and structural status of the protein. We have clearly observed the alteration in native conformation and perturbation in the secondary structure of  $\alpha_2\text{M}$  on the exposure to small antioxidant molecule-gallic acid. Moreover our studies with antioxidant enzymes and scavengers suggest that antiproteolytic activity of sheep  $\alpha_2\text{M}$  was significantly reduced due to oxidative modifications mediated by superoxide anion and hydrogen peroxide generated from photo-illumination of gallic acid. We have shown that this inactivation primarily involves subtle structural changes in  $\alpha_2\text{M}$ . Conformational and structural alterations induced in  $\alpha_2\text{M}$  by gallic acid on photo-illumination

were illustrated by various biophysical techniques (UV/visible absorption spectroscopy, fluorescence quenching studies, synchronous measurements, FTIR, CD and ITC). Any inactivation of  $\alpha_2\text{M}$  by ROS will compromise the antiproteinase shield and will lead to tilting of proteinase-antiproteinase balance in the favour of proteinases which is a hallmark of various diseases such as atherosclerosis, emphysema and rheumatoid arthritis [22] although additional studies maybe required to document this fact in our case.

**Acknowledgements** TS is thankful to DBT, New Delhi, for providing DBT-SRF fellowship. MKZ is thankful to UGC-MANF for providing fellowship in the form of SRF.

## Compliance with Ethical Standards

**Conflict of Interest** None.

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