

Ameliorative Effect of Quercetin against Modification Induced by 5-Fluorouracil on Alpha-2-Macroglobulin

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ABSTRACT

Introduction: Alpha-2-macroglobulin (α_2 M) is large glycoprotein, found in the plasma of vertebrates and invertebrates, which plays a major role in regulation and transport. In this study, we report the interaction of 5-fluorouracil (5-FU) with α_2 M and the protective effect of quercetin against 5-FU induced modification of α_2 M. **Methods:** Various biochemical and biophysical methods and techniques were employed to determine the binding interaction between α_2 M-5-FU and the preventive effect of quercetin on 5-FU induced modification of α_2 M. Fluorescence microscopy was performed to analyze the formation of free radicals by 5-FU and to examine the scavenging effect of quercetin. **Results:** Our results of antiproteinase activity assay show that the 5-FU- α_2 M interaction causes functional inactivation of sheep α_2 M, while the 5-FU- α_2 M complex after exposure to quercetin regains its native conformation. Intrinsic fluorescence results suggest an increase of 5-FU quenching in the fluorescence intensity, and when the mixture was incubated with quercetin the α_2 M-FU-quercetin mixture showed the same intensity as that of native protein. The absorption spectra of 5-FU- α_2 M complex suggest the formation of a complex leading to increase in absorbance. CD and FTIR spectroscopy show 5-FU causes secondary structural alteration of α_2 M and quercetin provides a protective role against structural modification. **Conclusion:** Our study suggests that 5-FU produces reactive oxygen species (ROS) in the presence of light and compromises the integrity of α_2 M. Quercetin quenches the ROS formed by 5-FU and helps the protein to maintain its native conformation. We have demonstrated the protective effect of quercetin for the first time against the toxicity induced by 5-FU on α_2 M.

Key words: 5-Fluorouracil, alpha-2-macroglobulin, circular dichorism, flavonoids, FTIR, quercetin, reactive oxygen species

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INTRODUCTION

5-fluorouracil (5-FU), a fluorinated pyrimidine analogue, is an anticancer drug and is an analogue of uracil containing fluorine at C-5 position instead of hydrogen atom¹. 5-FU can be incorporated into DNA and RNA due its structure and the fact that it interferes with nucleoside metabolism, which may lead to cell death and cytotoxicity². 5-FU is found to be a beneficial drug used for the treatment of tumours of the head, breast and neck³. It is also an effective drug to treat cancers of the gastrointestinal tract, stomach, cervix and colon⁴⁻⁷. However, it is also associated with toxicity, the most common being acute coronary syndrome^{8,9}, and the administration of 5-FU to humans can induce cardiotoxic effects such as cardiomyopathy and vasospastic angina¹⁰⁻¹⁴. The interaction of anticancer drugs with proteins is very effective in pharmacology and is considered to be important for the administration of drugs in the human body. Sheep alpha-2-macroglobulin (α_2 M) is a large tetrameric glycoprotein of 630 kDa¹⁵, containing

identical subunits joined by non-covalent disulphide bonds^{16,17}. The α_2 M has been isolated from various sources such as haemolymph of invertebrates, plasma of vertebrates and the egg white of birds and reptiles¹⁸⁻²². It is a major antiproteinase found in the blood and tissues and binds to various cytokines and growth factors²³ and is also responsible for the binding and transport of human growth hormone²⁴. It is a classic proteinase inhibitor and can inhibit all proteinases irrespective of their specificity and catalytic mechanism^{25,26}; however, it does not inactivate them, which makes it unique. After inhibition, it obstructs the entry of substrates (large molecular weight) to the active site²⁷.

Flavonoids are the compounds of plant origin and possess antioxidant and anti-inflammatory properties. Quercetin provides a protective effect against free radicals and also plays a vital role in the reduction of oxidative stress²⁸⁻³². The aim of this study is to investigate the antioxidant effect of quercetin and its protective effect on 5-FU-induced modification of α_2 M. Several studies show that 5-FU produces

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free radicals in the presence of light, which disrupts the integrity of proteins³²⁻³⁷. When 5-FU interacts with α_2M in the presence of light it induces a change in α_2M both structurally and functionally. In the present study, we provide evidence for the binding of 5-FU with α_2M . Moreover, we also provide information regarding the protective effect of quercetin against the toxicity induced by 5-FU on α_2M .

METHODS

Materials

Trypsin, soya bean trypsin inhibitor (STI), N-Benzoyl-DL-arginine-p-nitroanilide (BAPNA) and Sephacryl 300HR, quercetin, 5-FU and quercetin were obtained from Sigma-Aldrich, India. All reagents used were analytical grade and commercially available.

Methods

α_2M antiproteinase activity

To analyse the change in the functionality of α_2M , antiproteinase assay was performed. The activity profile of protein was measured with increasing concentrations of 5-FU in the presence of light. To determine the protective effect of quercetin, it was preincubated with protein and then treated with 5-FU³⁸. The reaction mixture consisted of 1 ml sample in 50 mM sodium phosphate buffer (pH 7.4) at room temperature (22 ± 1 °C). After incubation for 1 hr, 0.161 nmol trypsin was added into the mixture and incubated for 15 min at 37°C. After 15 min incubation, 0.45 nmol of soybean trypsin inhibitor (STI) was added into the reaction mixture and the incubation was continued for additional 15 min at 37°C. Finally, 2.28 μ mol of N-Benzoyl-DL-arginine-p-nitroanilide (BAPNA) was added and absorbance was observed at 410 nm.

Intrinsic fluorescence measurements

The fluorescence spectra for native α_2M and 5-FU and quercetin were recorded on a Shimadzu RF-5301 spectrofluorometer (Tokyo, Japan) in a 10 mm path length quartz cell. The excitation wavelength was set at 280 nm and the emission spectra were recorded in the range of 300-400. The concentration of protein in the sample was 10 μ M with varying concentration of 5-FU and the concentration of quercetin was 50 μ M in the aliquot.

Synchronous fluorescence analysis

Synchronous fluorescence was recorded on a Shimadzu RF5301 spectrofluorophotometer, Japan. The

wavelength range (250 – 400 nm) of fluorescence spectra selected for synchronous scanning was recorded at $\Delta\lambda$ of 15 nm and 60 nm for tyrosine and tryptophan, respectively.

UV absorption spectroscopy

UV spectrum of native α_2M (10 μ M) and α_2M incubated with quercetin (50 μ M) with increasing concentration of 5-FU were recorded between 250 – 350 nm on a Shimadzu UV visible Spectrophotometer UV-1700 using a cuvette of 1 cm path length³⁹.

FTIR spectroscopy

FTIR spectroscopy was performed on an Interspec 2020 FTIR spectrometer. The samples were prepared by dissolving native α_2M (20 μ M) and α_2M incubated with quercetin and 5-FU in 20 mM sodium phosphate buffer, pH 7.4. The spectra of native α_2M incubated with varying concentration of 5-FU and quercetin (50 μ M) were analyzed. Amide I ($1600 - 1700$ cm^{-1}) is the region which is usually analyzed to confirm the change in the native structure of the protein. The scanning wave number was $1000 - 4000$ cm^{-1} ⁴⁰.

CD measurements

CD measurements were recorded on a JASCO-J815 spectropolarimeter equipped with a Peltier-temperature controller. Wavelength scan for native α_2M (20 μ M) and quercetin with varying concentration of 5-FU were carried out. The temperature was maintained at 25°C and the speed was fixed at 100 nm/min with a response time of 1s. Each spectrum was the average of 3 scans and was recorded in the wavelength range of 200 – 250 nm using quartz cuvette of 0.1 cm path length.

Measurement of superoxide generation by 5-FU

The superoxide species ($\text{O}_2^{\cdot-}$) generation was estimated by the nitro-blue-tetrazolium (NBT) assay⁴¹. A typical assay mixture was used containing 50 mM sodium phosphate buffer (pH 8.0), 300 μ M NBT, 100 μ M EDTA and 0.06% Triton-X-100 and α_2M (20 μ M) treated with varying concentration of 5-FU (10 – 50 μ M). The absorbance was recorded at 560 nm at different time intervals, against a blank which was without 5-FU.

Fluorescence microscopy

Fluorescence microscopy was utilized to determine the formation of reactive oxygen species (ROS). De-

termination of ROS was performed using the dichlorofluorescein (DCF) method⁴². Protein was preincubated with quercetin for 1 hr, treated with 5-FU and incubated in the presence of white light. Then the sample was incubated with 10 μ M dichloro-dihydrofluorescein diacetate (DCFH-DA) for 1 hr at 37°C. The treated protein was placed on glass coverslips and visualized under a fluorescent microscope at 40 x magnification.

Statistical Analysis

Results were shown as the mean and the standard deviation (SD values, with n=3 as the number of independent experiments).

RESULTS

Antiproteinase activity

The activity assay of native α_2 M with varying concentration of 5-FU was measured in order to check the functional status of the protein. Firstly, we measured the antiproteinase activity of protein incubated with the drug in the presence of white light. Our results show that the activity of protein decreases with increasing concentration of 5-FU (10 – 50 μ M). According to **Figure 1a**, the residual activity was found to be 87% at 10 μ M 5-FU. When the concentration of the drug (50 μ M) was increased, the remaining activity was found to be 48%. According to previous studies, 5-FU is known to generate free radicals in the presence of white light⁴³. Therefore, the anti-proteolytic potential of the protein was compromised, which may be due to the formation of free radicals by the drug leading to changes in the functionality of the protein. We measured the protective effect of quercetin against the toxicity induced by 5-FU, since it is a flavonoid which is known to scavenge free radicals⁴⁴. When the protein was preincubated with quercetin and then treated with 5-FU in the presence of light the resultant activity decreased, but not as much compared to 5-FU alone. Our results suggest that the residual inhibitory activity at 50 μ M 5-FU was 59% when the protein was preincubated with quercetin (**Figure 1b**). Therefore, quercetin provides a protective role against 5-FU since it may scavenge free radicals that are generated by the drug.

Intrinsic fluorescence measurements

Intrinsic fluorescence is mainly used to determine the conformational changes in the native form of the protein. The fluorescence spectra were recorded for the protein (20 μ M) with increasing concentration of 5-FU (10 – 50 μ M). The protein was excited at 280 nm

and emission spectra were taken in the range 300 - 400 nm. The emission maximum ($\lambda_{max}/\lambda_{em}$) for native α_2 M was found to be 328 nm, as shown in **Figure 2a**. It is quite clear from the figure that with increasing concentration of 5-FU, the fluorescence intensity decreases continuously with a blue shift of 3 nm. The decrease in the intensity is due to the quenching in fluorescence due to the interaction between the protein and the drug. The quenching occurs mainly due to three intrinsic fluorophores in the protein *i.e.*, phenylalanine, tyrosine and tryptophan⁴⁵. The change in the fluorescence intensity can be correlated to the change in the structure of α_2 M as the ligand binds to it⁴⁶, which may also alter the function of the protein. Many *in vitro* studies suggest that quercetin can reduce the toxicity induced by anticancer drugs and provide a protective effect against free radical induced damage³¹. To evaluate the protective effect of quercetin against 5-FU-induced toxicity on α_2 M, intrinsic fluorescence was recorded. The protein was first preincubated with quercetin and then treated with the drug in the presence of white light, intrinsic fluorescence was then recorded. As the results suggest, with varying concentrations of 5-FU the fluorescence intensity decreases, but when the protein was preincubated with quercetin, less quenching occurs (**Figure 2b**). So, we can conclude that in the presence of quercetin, α_2 M regains its native structure which was damaged by the drug.

Synchronous fluorescence analysis

Synchronous fluorescence was performed to study the changes in the molecular environment in the vicinity of the chromophore. The spectral analysis provides information about the conformational changes around the tyrosine and tryptophan residues of the protein. The effect of fluorescence analysis of 5-FU on α_2 M is shown in **Figure 3**. With the addition of drug, there was a decrease in the fluorescence intensity with a shifting of emissions towards a longer wavelength from 341 to 344 nm for tryptophan residues and towards a shorter wavelength from 309 to 305 nm for tyrosine residues. This decrease in the fluorescence intensity with varying concentration of 5-FU suggests changes in the surrounding microenvironment around tyrosine and tryptophan residues with a decrease in hydrophobicity around both residues⁴⁷.

UV absorption spectroscopy

UV spectra was analyzed to determine the structural change in the native protein and to study the formation of a complex between protein and ligand⁴⁸. It

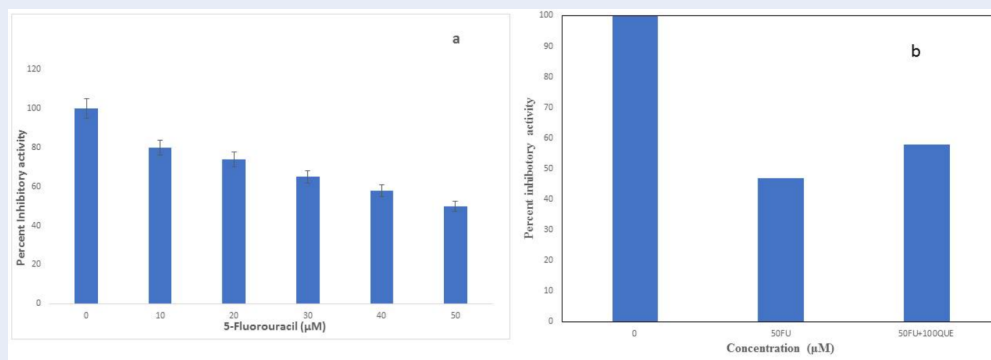


Figure 1: Percent inhibitory activity of α_2M (a) α_2M was incubated with increasing concentration of 5-FU in light assayed for antiproteolytic activity (b) α_2M was preincubated with quercetin (100 μM) and then incubated with 5-FU (50 μM) in visible light then assayed for antiproteolytic activity (Paired t for conc - %IA, 95%. CI for mean difference: -84.2276, -8.1058. T-test of mean difference = 0 (vs not = 0): t-value = -3.12, p-value = 0.026).

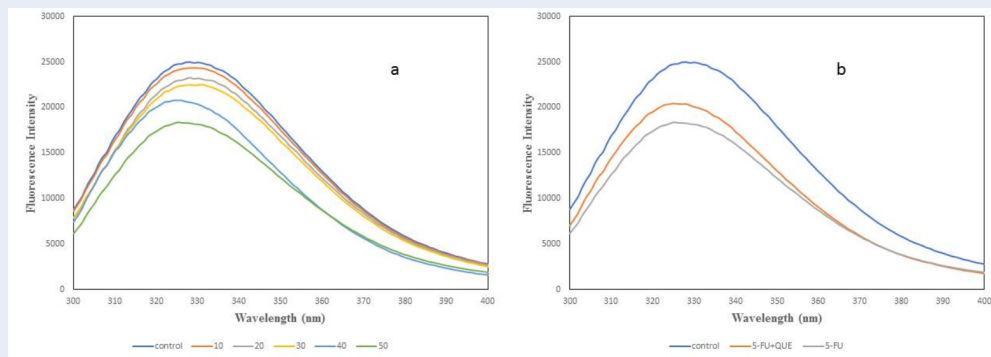


Figure 2: Fluorescence spectroscopy (a) fluorescence spectra of native α_2M and 5-FU treated in light (b) fluorescence spectra of α_2M preincubated with quercetin (100 μM) and then treated with 5-FU (50 μM) in the presence of visible white light.

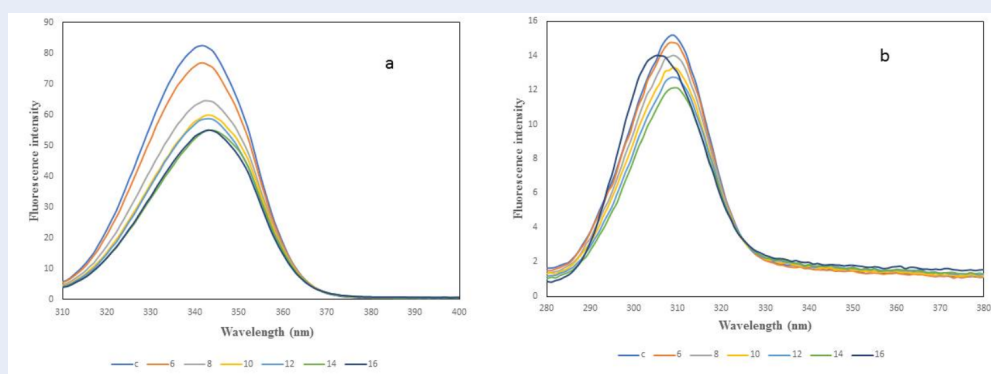


Figure 3: Synchronous fluorescence spectra of α_2M with $\lambda = 15$ nm (A) and $\lambda = 60$ nm (B) in the presence of 5-FU (6 - 16 μM).

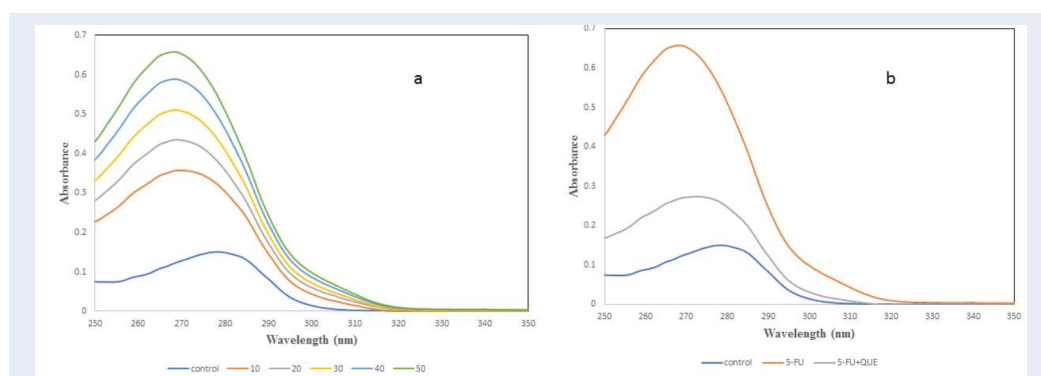


Figure 4: UV spectroscopy (a) α_2 M was incubated with increasing concentration of 5-FU (10 - 50 μ M) in light; (b) α_2 M was preincubated with quercetin (100 μ M) then incubated with 5-FU (50 μ M).

is a classical technique to determine the modification in the microenvironment of amino acid residues, particularly tryptophan, resulting in the change in the spectra or a shift in maximum absorbance/wavelength (λ_{max})⁴⁵. To examine the change in the native structure of α_2 M with varying concentration of 5-FU, spectra was recorded between 250 – 350 nm (Figure 4a). As shown in Figure 4, the λ_{max} for native α_2 M was found to be 278 nm, but with increasing concentration of 5-FU, the absorbance increases and a blue shift (10 nm) was observed. The blue shift indicates that the interaction of drug with α_2 M causes some structural changes in the native form of protein. It is well-known that 5-FU forms free radicals in the presence of white light that may cause damage or change in the protein. So, quercetin was used as a protective agent to scavenge the free radicals formed by the drug. α_2 M was preincubated with quercetin and then treated with 5-FU in the presence of light. When α_2 M was preincubated with quercetin, the effect of drug was less compared to drug alone and λ_{max} was found to be 275 nm (Figure 4b). Our results suggest that quercetin scavenges free radicals formed by drug and prevents oxidative damage and/or restores the native structure of protein.

Measurement of superoxide generation by 5-FU

5-FU was subjected for photo-illumination in order to measure the free radical(s) generated by it. The free radical generated by drug was measured by the NBT reduction assay. Figure 5a depicts the generation of superoxide anions ($O_2^{\cdot-}$) with increasing concentration of 5-FU (10 – 100 μ M). α_2 M was preincubated with quercetin (100 μ M) and then exposed to 5-FU for 1 hr in light. According to Figure 5b, quercetin decrease the formation of superoxide anion as compared

to 5-FU alone. So, the results suggest that quercetin has antioxidant activity against the formation of free radicals by 5-FU.

FTIR spectroscopy

The FTIR spectroscopy was utilized to determine the change in the secondary structure of protein. Amide I band was analyzed as it is the most important protein band for the analysis of possible structural changes. The amide I band displays in the wave number range 1600 – 1700 cm^{-1} governed by the stretching of the C=O (70 – 85%) and minor contribution of C-N groups (10 – 20%). Figure 6 shows the FTIR spectra of native α_2 M along with 5-FU and quercetin. The amide I band for native α_2 M was centered at around 1635 cm^{-1} which is characteristic of β -sheet structure³⁸. However, upon incubation with 5-FU, the amide I band was at 1635 cm^{-1} , but the intensity decreases with varying concentration which implies the loss of protein native conformation. The native conformation of α_2 M is lost, which affects the functional activity of the antiproteinase inhibitor suggesting that 5-FU causes structural changes in the native conformation of protein, which was confirmed by the antiproteinase activity results. In order to determine the preventive effect of quercetin, the protein was incubated with 5-FU. Figure 6 shows that the band for α_2 M preincubated with quercetin was found to be close to that of native protein. Hence, quercetin scavenges the free radicals from the drug and provides a protective role against the oxidative modification of α_2 M.

CD measurements

CD spectroscopy was performed to analyse the structural transitions in the structure of protein since it

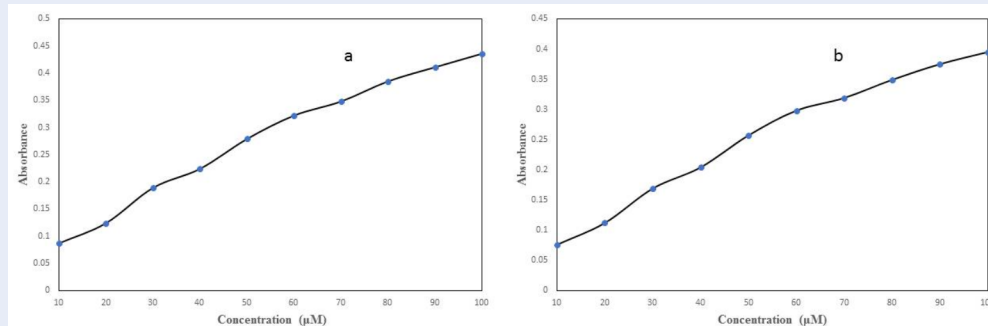


Figure 5: Generation of superoxide anion (a) 5-FU (10 – 10 μM) in the presence of light (b) α₂M was pre-incubated with quercetin (100 μM) and then exposed to 5-FU (10 – 10 μM) in light for 1 hr.

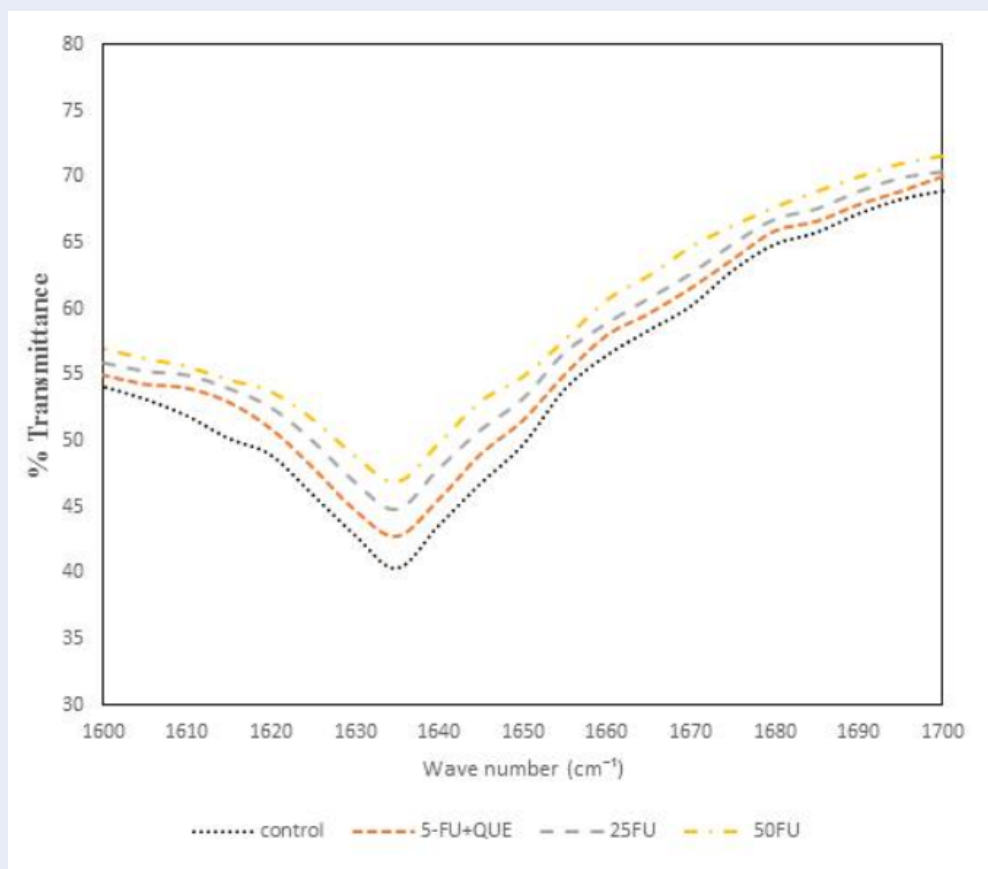


Figure 6: FT-IR spectra of native α₂M and treated with 5-FU and quercetin in light.

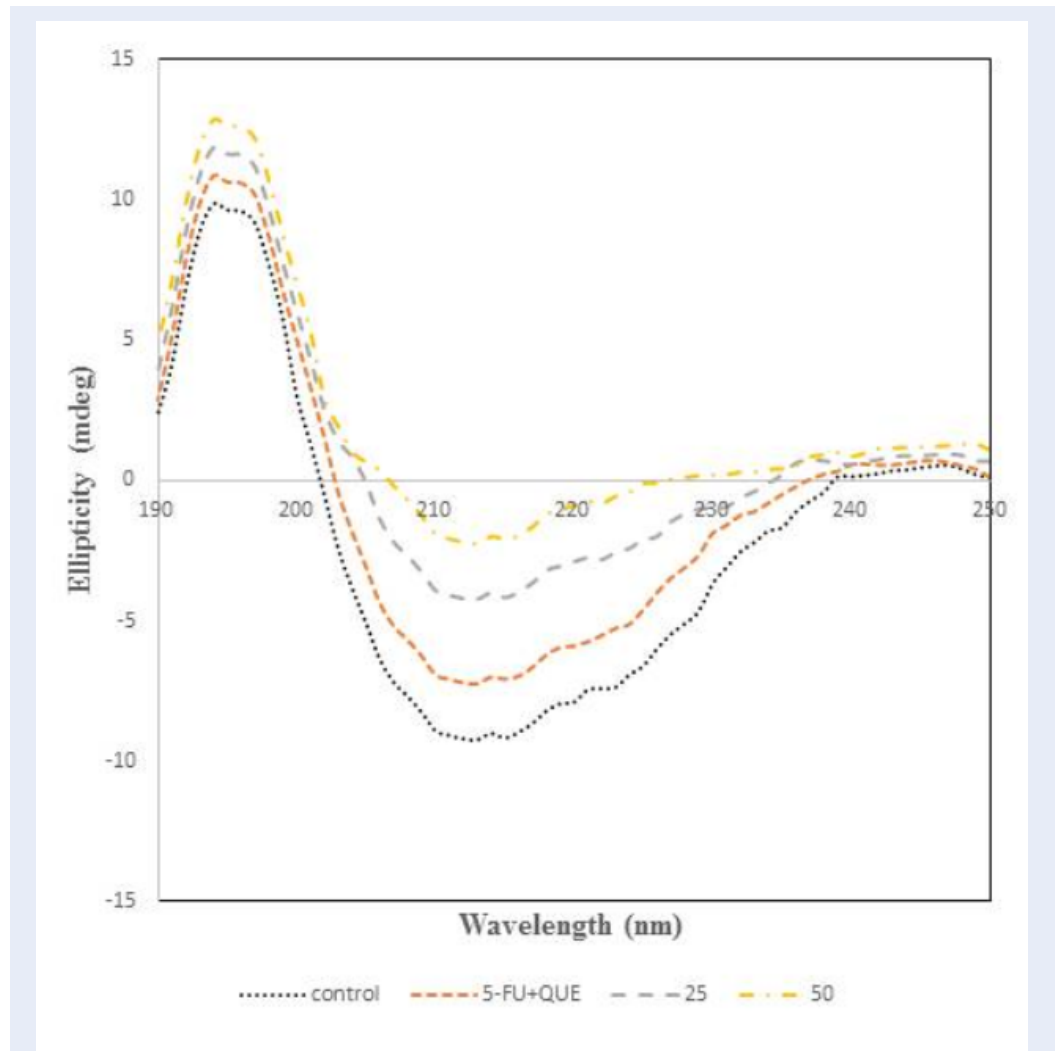


Figure 7: Far-UV circular dichroism spectra of native α_2M , treated with 5-FU and quercetin.

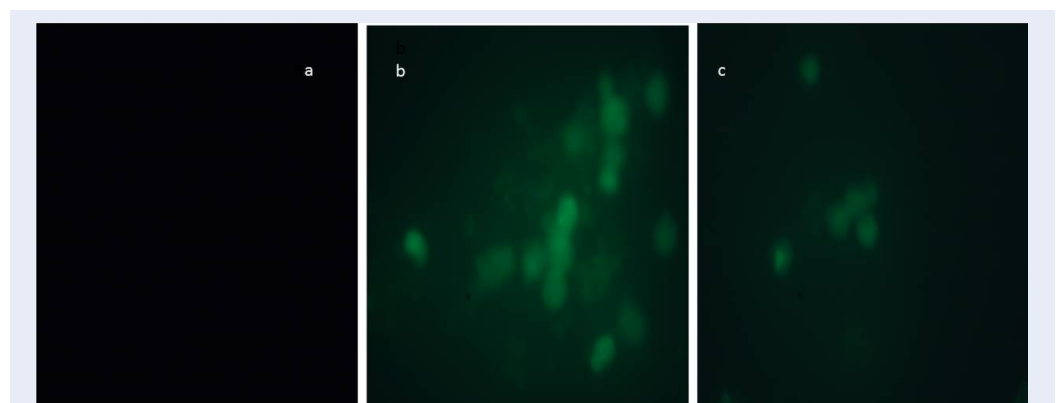


Figure 8: Representative fluorescent microscopic images (a) native α_2M , (b) treated with 5-Fluorouracil (50 μM) and (c) treated with quercetin (100 μM) and 5-FU (50 μM).

gives information regarding the secondary structural changes⁴⁹⁻⁵¹. The CD spectra of α_2 M incubated with 5-FU and quercetin are shown in **Figure 7**. The peak of native protein was found at 215 nm which is a characteristic feature of β -sheet structure^{38,39}. However, varying the concentration of 5-FU leads to changes in the far UV-CD spectra resulting in the increase, which suggests the loss of β -sheet content in native α_2 M. So, the formation of free radicals by the drug causes structural distortion in the native form of protein. Thus, it can be said that the loss in β -sheet content of the protein alters the biological activity of the protein. In order to identify the protective effect of quercetin, it was incubated with α_2 M and 5-FU and it was found that α_2 M preincubated with quercetin shows spectra very similar to that of native protein.

Fluorescence microscopy

The effect of drug on the native protein and effect of quercetin on 5-FU treated protein was examined. As depicted in **Figure 8a**, the native protein shows no fluorescence which indicates no free radicals are present in the sample. 5-FU is known to form free radicals in the presence of white light which can damage the native conformation of protein. When α_2 M was incubated with 5-FU (50 μ M) it showed bright fluorescence representing the production of free radicals (**Figure 8b**). When α_2 M was preincubated with quercetin and then treated with 5-FU, a weaker fluorescence was observed, which shows a reduction in the formation of free radicals (**Figure 8c**).

DISCUSSION

The flavonoids are the polyphenolic compounds of plant origin found in vegetables, fruits, red wine, tea, *etc.* and possess antioxidant and anti-inflammatory properties²⁸⁻³¹. Quercetin provides a protective effect against free radicals induced oxidative stress and possesses many beneficial effects against liver disease that may be caused by the endotoxins³²⁻³⁷. In the present study, the interaction of 5-FU with α_2 M was examined for the first time. Quercetin was incubated with 5-FU treated α_2 M and antiproteinase activity assay was performed to measure the functional activity of the protein. 5-FU causes structural and functional changes in the native conformation of α_2 M and quercetin was used to prevent the toxic effects of the drug. The results obtained from the intrinsic fluorescence and absorption spectroscopy show that 5-FU causes conformation changes in the native form of protein due to the formation of free radicals and quercetin provided a protective role. CD

and FTIR spectroscopy were used to analyze the secondary structural changes in the protein treated with the drug and it regained its native conformation in the presence of quercetin. Fluorescence microscopy was analyzed to study the formation of free radicals by 5-FU, the results show that 5-FU forms free radicals and quercetin provides a scavenging role. On the other hand, when the α_2 M-5FU mixture was treated with quercetin, the protein regains its native conformation. Our study provides an insight into the 5-FU- α_2 M interaction and the scavenging role of quercetin.

CONCLUSIONS

In the present study, we reported the interaction of 5-FU with α_2 M and the protective effect of quercetin against 5-FU induced modification of α_2 M. Various biochemical and biophysical methods were employed to determine the binding between α_2 M-5-FU and the protective effect of quercetin on 5-FU induced modification of α_2 M. Photoilluminated 5-FU produces ROS which compromises the structural and functional integrity of α_2 M. Quercetin quenches the ROS produced by 5-FU and helps the protein to maintain its native conformation. Therefore, we demonstrated the protective effect of quercetin for the first time against the toxicity induced by 5-FU on α_2 M.

ABBREVIATIONS

5-FU: 5-fluorouracil, **α_2 M**: alpha-2-macroglobulin, **CD**: circular dichroism, **FTIR**: fluorescence transform infra-red spectroscopy, **ROS**: reactive oxygen species

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AUTHOR'S CONTRIBUTIONS

SSA: collected data and performed analysis; acquisition, analysis and interpretation of data; drafted the manuscript; HA: wrote the paper; drafted the manuscript; critically revised the manuscript; SA: performed the analysis; FHK: conceived, conceptualized and designed the study; gave final approval.

FUNDING

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AVAILABILITY OF DATA AND MATERIALS

Data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was conducted in accordance with the amended Declaration of Helsinki. The institutional review board approved the study, and all participants provided written informed consent.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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