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Research Article

Synthesis of novel iron chelating and heme-interacting acridone derivatives to prevent free heme- and iron-mediated protein oxidation

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Abstract

Objectives: Various diseases are associated with the accumulation of oxidized proteins. In certain pathological conditions, the breakdown of hemoproteins, such as hemoglobin, leads to the formation of heme. Heme possesses pro-oxidant properties, which contribute to oxidative stress, inflammation, and protein degradation. Furthermore, the presence of reduced transition-metal ion Fe^{2+} can lead to protein degradation and the generation of harmful hydroxyl radicals (•OH). It would be beneficial to develop a substance that can counteract these processes by detoxifying free heme, chelating Fe^{2+}, and exhibiting antioxidant activity through the scavenging of •OH, thereby preventing protein oxidation.

Methods: Two new acridones, 2-chloroacridin-9(10H)-one (a) and 2-fluoroacridin-9(10H)-one (b), have been synthesized. These compounds have the ability to interact with heme and bind to free iron. The effectiveness of their interaction with heme and their iron-binding properties was assessed through optical Soret spectroscopy. To determine the protective effects of these newly synthesized acridones on protein degradation induced by heme and iron, a standard protein called bovine serum albumin was employed. In addition, the in vitro antioxidant activity of the synthesized compounds was evaluated using two different assays: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and the ferric reducing antioxidant power assay.

Results: The acridones demonstrated exceptional abilities in chelating iron, interacting with heme, and exhibiting antioxidant activity. The specific substitutions of chlorine and fluorine atoms in synthesized acridones, respectively, differentiate them from other acridones in terms of their distinct electronic, steric, and chemical characteristics that influence their interactions with iron and heme molecules. Moreover, these compounds effectively inhibited protein degradation induced by heme and the production of •OH resulting from the Fenton's reaction involving Fe(II) and hydrogen peroxide (H_2O_2).

Conclusion: From the above observation, it is evident that, acridones have the potential to treat disorders by preventing the oxidation of proteins by free heme, iron, and •OH.

Keywords: Acridones, antioxidant activity, heme-interaction, iron chelator, protein oxidation

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One of the primary causes of oxidative stress in biological systems is free heme and iron [1–3]. A prosthetic group called heme attaches to many different proteins and enzymes, including cytochromes and hemoglobin. Heme can, however, be liberated from its protein partners in pathological circumstances, such as hemolysis, inflammation, or ischemia-reperfusion injury, and has harmful consequences for cells and tissues [4–6]. Reactive oxygen and nitrogen species, which can harm lipids, proteins, and DNA, can be produced by heme through catalysis [7–10]. Furthermore, heme can activate numerous signaling pathways linked to cell death and inflammation, as well as increase the expression of pro-inflammatory cytokines and adhesion molecules [2, 11].

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Numerous biological functions, including DNA synthesis, electron transfer, and oxygen transport, depend on iron [12, 13] By taking part in the Fenton reaction, which produces incredibly reactive hydroxyl radicals (•OH) from hydrogen peroxide (H_2O_2), excess iron can, however, also lead to oxidative stress [10, 14]. Many diseases, including hemochromatosis, thalassemia, and sickle cell disease, can result in iron overload. Organ failure and tissue damage can result from iron-mediated oxidative stress, particularly in the liver, heart, and brain [15–18].

To avoid their harmful effects on biological macromolecules, new chemicals that can scavenge unbound heme and iron are needed. The class of heterocyclic chemicals known as acridone derivatives has demonstrated a variety of pharmacological actions, including antibacterial, antiviral, antimalarial, and anticancer effects [19–25]. In addition, several acridone derivatives have been shown to interact with heme and have the ability to chelate iron, making them prospective candidates for modifying heme and iron homeostasis [26]. This work investigated the ability of a variety of novel acridone derivatives to chelate iron and interact with heme by synthesizing them with various substituents on the ring system. *In vitro* evaluations have also been made of protective effects against free heme and iron-mediated protein oxidation. In addition, these acridones exhibit antioxidant action *in vitro*.

Materials and Methods

Materials

Hemin chloride (Sigma, St Luis, MO, USA), Eaton's reagent (Aldrich, St Luis, MO, USA), 4 chloro aniline (Alfa asear, Tewksbury, USA), 4 fluro aniline (Alfa asear, Tewksbury, USA), 2-Chloro benzoic acid (Alfa asear, Tewksbury, USA), Anthranilic acid, and 3-chlorobenzenamine (SRL, Kolkata, India). Other all chemicals and reagents were obtained from Aldrich (Milwaukee, WI), Lancaster (England) or Spectrochem (India) and were used without further purification.

Chemical synthesis

2-chloroacridin-9(10H)-one (A) and 2-fluoroacridin-9(10H)-one (B)

A mixture of 4-halobenzenamine (1 eqv), 2-chloro benzoic acid (1eqv), K_2CO_3 (1.2 eqv), and powdered Cu (catalytic) in isopentyl alcohol was heated under reflux for 4–5 h. Then, 1M K_2CO_3 was added to this solution, and the insoluble particles were removed by filtering. The aqueous layer was then extracted with chloroform, acidified with 2M HCl, and dried over Na_2SO_4. The crude residue was vaporized to remove the solvent, and the alumina column was used to chromatograph the crude residue to produce the pure chemical (I).

Compound I was heated with Eaton's reagent (8 mL) at 80°C for 50 min until the completion of the reaction as indicated by thin layer chromatography. After allowing it to reach room temperature, the reaction mixture was progressively added to the saturated aqueous $NaHCO_3$ solution. The solution was then evaporated to dryness in a vacuum before being extracted with chloroform and dried over Na_2SO_4. To obtain the pure products A and B, the impure residue was purified using alumina column chromatography (Fig. 1) [26].

Binding of acridones with heme (ferriprotoporphyrin IX)

Heme (10 µM) in 100 mM phosphate buffer with a pH of 7.4 is present in the assay system, which has a total capacity of 1 mL. Small amounts of acridones in various concentrations (2, 4, 6, 8, 10, 12, 14, 18 and 20 µM) were gradually added to the heme solution in the sample cuvette while also adding the same volume of dimethyl sulfoxide (DMSO) to the reference cuvette (acridones were dissolved in DMSO). The assay system is the same for the control group but without acridones. The absorbance of the optical Soret spectrum was measured in quartz cells with a 1 cm light path in a Perkin Elmer Lamda 15 UV/VIS spectrophotometer at a temperature of 25±1is°C to track the binding capacity of acridones at various concentrations (2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 µM) to heme. Before recording the spectrum, the ingredients were well blended. Nonlinear curve-fitting techniques were used to analyze the generated titration curves. Nonlinear curve-fitting techniques were used to analyze the generated titration curves [26].

Iron chelating activity *in vitro*

The assay system contains Fe (II) (10 µM) in 20 mM phosphate buffer at pH 7.4 in a total volume of 1 mL. Small amounts of 2,4,6-Tri(2-pyridyl)-s-triazine(TPTZ) (20 µM solution) and various concentrations (500 nM, 1, 10, 50, 80, and 100 µM) of acridones were added to the Fe (II) solutions in the sample cuvette at the same time as adding the equivalent volume of DMSO to the reference cuvette (acridones were dissolved in DMSO). The assay system is the same for the control group but without acridones. A well-known iron chelator called desferrioxamine was used as a positive control. By measuring the absorbance of the Fe (II)-TPTZ complex immediately after each addition in the quartz cells of a 1 cm light-path in a Perkin Elmer Lamda 15 UV/VIS spectrophotometer at 25±1°C, the ability of acridones to chelate iron at varied concentrations was observed. Before the spectrum was recorded, the components were thoroughly blended [27].

Measurement of heme and iron-mediated protein oxidation (carbonyl formation)

In this assay, a common protein called bovine serum albumin (BSA) was exposed to a system that produces reactive oxidants, such as heme or iron (II) and hydrogen peroxide (H_2O_2), as previously mentioned [2]. The test method included 10 µL, 10 mM 2,4 dinitrophenylhydrazine, 10 µL, 100 µM acridones in acetonitrile, 150 µL, 20 mM sodium phosphate buffer (pH 7.4), 10 µL, 5 µM heme or 10 µM ferrous sulphate, 10 µL, 10 µM H_2O_2, 10 µL,10 µg BSA, and a final volume of 200 µL. 1 µg of catalase was used to stop the reaction after an hour of room temperature incubation. BSA was precipitated using 25% TCA

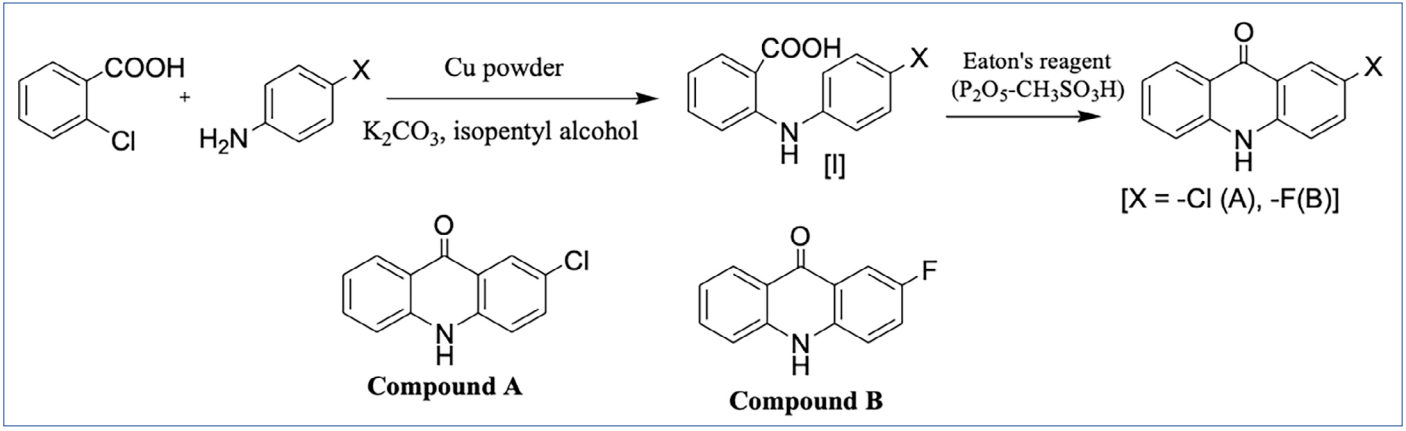


Figure 1. Synthetic scheme of acridone derivatives (A and B).

and then three times with an ethanol: Ethylacetate (1:1) solution. After that, the product was dissolved in 0.6 mL of a solution containing 6 M guanidine-HCl in 20 mM potassium phosphate, which was then centrifuged after being pH-adjusted to 2.3 using trifluoroacetic acid. Finally, the carbonyl concentration of the supernatant was measured at 362 nm [28].

Determination of *in vitro* antioxidant property of acridones by ferric-reducing antioxidant power (FRAP) assay

A 96-well microplate was used for the test. The 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ) solution was made by mixing 10 mL of acetate buffer (200 mM, pH-3.6), 1 ml of TPTZ solution (10 mM in 40 mM HCl), and 1 ml of ferric chloride solution (20 mM in distilled water) together. For one hour, the mixture was heated to 37°C in a water bath. The under researched acridones were dissolved in methanol at various concentrations (0.5,1,5,10 and 20 µM). Each well was filled with 175 µL of freshly made FRAP solution before receiving 25 µL of the acridones solution. To reduce error, each test was performed in triplicates. For 80 minutes, a microplate reader measured the 595 nm absorbance at various intervals. The absorbance of a mixture of 175 µL of FRAP solution and 25 µL of methanol, referred to as "blank," was measured concurrently, and its absorbance was subtracted from the samples' absorbance at each time interval to compute the absorbance change (ΔA). According to the formula, the FRAP value at time interval t (FRAP valuet) was determined [27, 28].

$$FRAP\ value_t\ (M) = (\Delta a_tT/\Delta a_tFe^{2+}) \times 10^{-5}$$

where Δa_tT is the absorbance change after the time interval t (6 and 65 min) relative to the tested acridones at the concentration of 10 µM and Δa_tFe^{2+} is the absorbance change of ferrous sulfate at the same concentration.

Free radical scavenging activity *in vitro* (2,2-diphenyl-1-picrylhydrazyl [DPPH] assay)

A stable-free radical called DPPH can take an electron or a hydrogen ion to transform into a stable diamagnetic compound. The DPPH radical's transformation from purple to yellow indicates that the antioxidants can scavenge it by donating hydrogen. The test setup contained 4 mL of DPPH (0.15 mM) in methanol (80% in water v/v) and 1 mL of Acridones solution at various concentrations (0.5, 1, 5, 10, and 20 µM). It was left to stand for 30 minutes without light at room temperature. Ascorbic acid was used as a positive control at the same concentration. The solution's absorbance was measured spectrophotometrically at 517 nm, and the antioxidant activity of acridones was estimated from the decrease in absorption [27, 28].

Results

Chemical synthesis

The chemical synthesis of acridones is outlined in Figure 1. Ullmann condensation between 2-chlorobenzoic acid and 4-halo aniline (x= -Cl, -F) in the presence of Cu powder and K_2CO_3 in isopentyl alcohol [26, 29], under reflux for 3 h, provided the 2 2-(4-halo phenylamino)benzoic acidin (I) 65% yield. Eaton's reagent [20, 30] was applied to compound I for 50 minutes at 80°C to produce the cyclized product 2-halo acridin-9(10H)-one in 68% yield. It is crucial to note that only 15% of the molecule [31] was produced when a similar cycloacylation procedure was conducted in the presence of polyphosphoric acid (PPA), as reported by others. In addition, the high viscosity of PPA makes reaction handling challenging for large-scale operations. The crude product was purified by column chromatography on alumina using 4% MeOH in $CHCl_3$ as eluents to give **A** in 68 % yield. R_f: 0.465 (8% MeOH in $CHCl_3$); ^{1}H NMR (300 MHz, DMSO-d_6), chemical shift(δ) 7.16–7.33 (m, 3H), 7.70 (t, 1H, *J*=6.9 Hz), 7.80 (d, 1H, *J*=7.8 Hz), 7.94 (d, 1H, *J*=8.1 Hz), 8.21 (d, 1H, *J*=7.8 Hz), 11.23 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6), 112.35, 117.15, 118.33, 120.58, 121.12, 121.28, 125.75 (2C), 131.86, 133.12, 140.69, 147.84, 176.61; MS (ESI) m/z calcd. for $C_{13}H_8ClNO$: 229.03; found: 252.02 $[M+Na]^+$.

Heme binding studies

The ability of these synthesized acridones to interact with heme has been examined. At pH 7.4, the interaction between the acridones and heme was studied. A large peak at 362 nm was observed in a solution of heme at pH 7.4, indicating that dimers

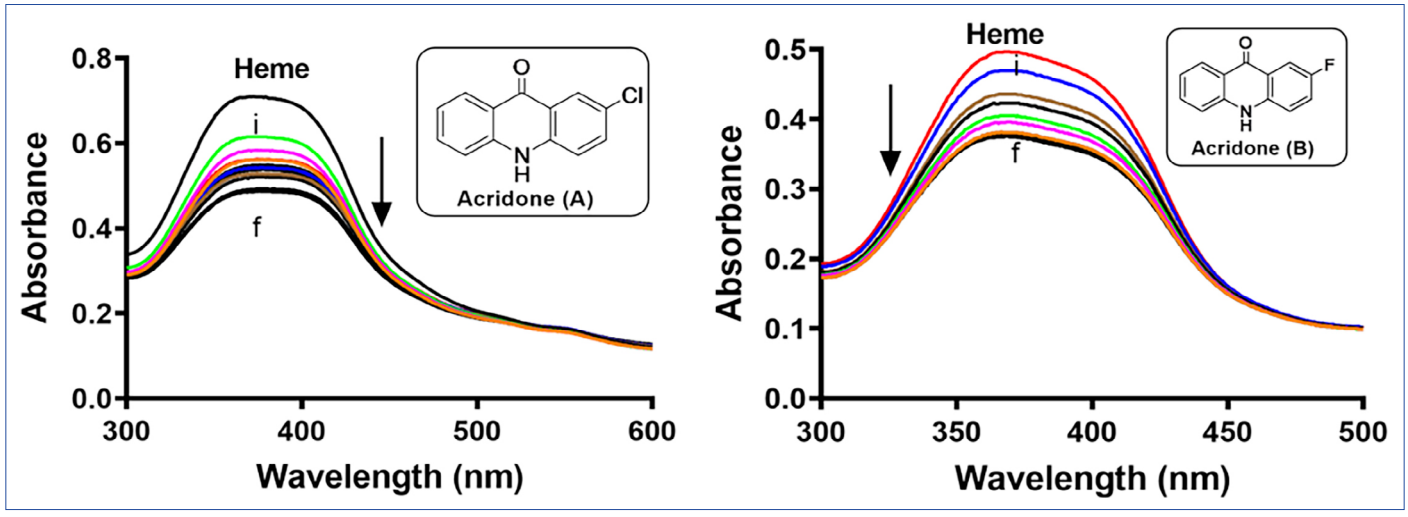


Figure 2. Interaction of acridone derivatives with heme. Optical Soret spectroscopy for acridones-hemin interaction at different concentrations of acridones (2–20 µM) (i, 2 µM; f, 20 µM).

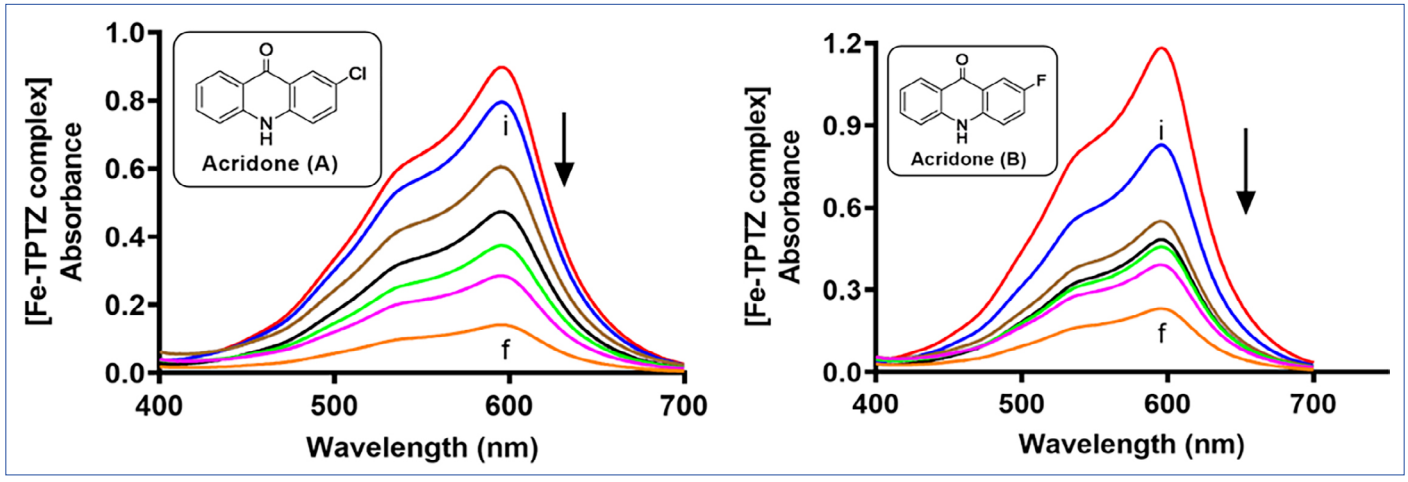


Figure 3. Iron-chelating activity of acridones. Spectroscopic analysis for acridones-Fe (II) interaction at different concentrations of acridones (500 nM-100 µM).

Fe-TPTZ: Iron-2,4,6-Tripyridyl-S-Triazine (TPTZ).

of the µ-oxo type or µ-hematin type predominate under our *in vitro* conditions [27, 32, 33]. Acridones were added, and this visibly disrupted the heme spectrum (Fig. 2). Acridones exhibit impressive binding abilities with heme, demonstrated by their high binding capacities. Acridone A has a binding capacity of $(2.1\pm0.15) \times 10^4$ M^{-1}, while acridone B has a binding capacity of $(2.32\pm0.09)\times10^4$ M^{-1}. Heme Soret molar absorptivity was reduced, and the Soret band was shifted to longer wavelengths, as a result of titration with acridones of increasing concentration (2 µM-20 µM) into the heme solution. Optical spectroscopy was used to quantify the binding of acridones to heme (Fig. 2). It is clear from the heme-binding analysis in Figure 2 that the acridones exhibit excellent heme-binding capacity.

Iron chelating activity

After assessing heme-interacting activity, the *in vitro* iron-chelating activity of acridones has been assessed. Using a tripyridyl triazine (TPTZ) assay, the iron-chelating ability was evaluated. In the presence of TPTZ, Fe (II) solution produced an absorbance at 595 nm (Fig. 3). Due to the development of the Fe (II)-TPTZ combination, this absorbance was achieved. Figure 3 shows that the absorbance of the Fe (II)-TPTZ complex drops when the acridones are introduced to the Fe (II) solution. This occurs as a result of acridones and Fe (II) chelating one another. The evidence suggests that acridones are effective iron chelators. Desferrioxamine, a common iron chelator, was used in this experiment, and the findings demonstrate that acridones have equivalent iron-chelating properties to that drug (data not shown).

Protective effect against heme- and iron-mediated protein oxidation (carbonyl formation)

Since acridones can chelate free iron and interact with heme, their protective role against iron-and heme-mediated protein

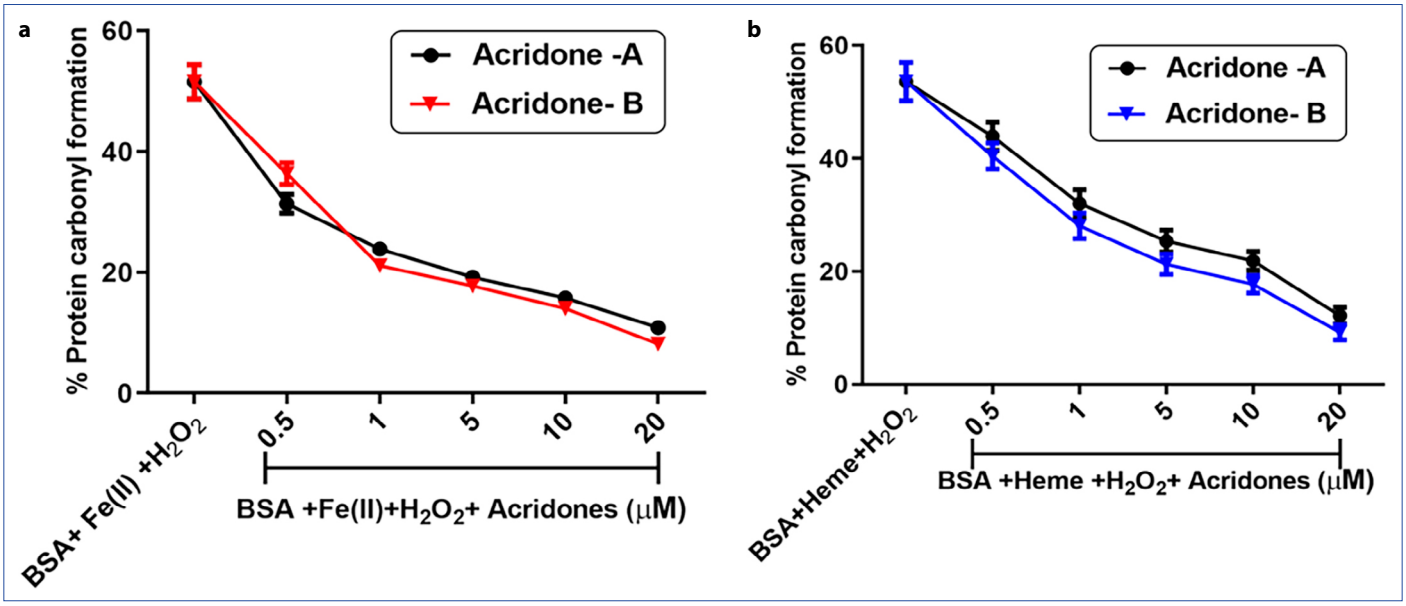


Figure 4. Protective effect of acridones on (a) iron-mediated (b) heme-mediated protein oxidation (carbonyl formation) markers for oxidative stress. BSA: Bovine serum albumin.

oxidation (carbonyl production) has been studied. Standard proteins such as BSA underwent substantial protein carbonyl formation due to oxidative alteration in the presence of Fe(II) and H_2O_2, as well as heme and H_2O_2 (measured by absorbance at 362 nm in the presence of DNPH) (Fig. 4). However, protein carbonyl production is greatly reduced in the presence of acridones.

Acridones offer protection against protein oxidation induced by iron and heme. Acridone A exhibits an IC_{50} value of 5.1±0.71 µM against iron-mediated protein oxidation and 4.9±0.78 µM against heme-mediated protein oxidation. Similarly, acridone B demonstrates an IC_{50} value of 4.8±0.42 µM for iron-mediated protein oxidation and 4.7±0.61 µM for heme-mediated protein oxidation. Heme and iron can catalyze oxidative reactions that lead to the generation of reactive oxidants and subsequent protein oxidation when present in excess or when released from proteins [34]. By successfully sequestering iron ions through their iron chelating activity, acridones decrease the amount of free iron that is available for fenton reactions. Acridones chelate iron, which decreases protein oxidation by reducing oxidative stress. This prevents iron-mediated reactive oxidants from being produced. This process protects the structural and functional integrity of proteins. Acridones also interact with heme, stabilizing it and preventing protein dissociation. This avoids the release of free heme, which can lead to protein carbonylation and oxidative stress [34]. Acridones inhibit the generation of heme-mediated reactive oxidants and ensuing protein oxidation, including the creation of carbonyl, by interacting with heme.

Antioxidant activity

FRAP assay

Since acridones protect heme- and iron-mediated protein oxidation (carbonyl formation), the antioxidant activity of acridones is need to be investigated. An antioxidant is a molecule that possesses both reducing and free radical scavenging activities. The reducing (antioxidant) ability of acridones was analyzed by the FRAP assay. The FRAP assay is based on the measurement of the ability of a substance to reduce Fe (III) to Fe (II); the greater the reducing ability, the greater the antioxidant property [28]. Antioxidants reduce the colorless Fe^{III}-TPTZ to a blue-colored Fe^{II}-TPTZ complex, which results in an increase in the absorbance at 595 nm giving a FRAP value. A higher FRAP value indicates greater reducing (i.e., antioxidant property) ability of the compound. Absorbance changes at 595 nm were measured at different time intervals (Fig. 5) and at different concentrations of acridones (0.5, 1, 5, 10, and 20 µM) (Fig. 5). Results (Table 1) clearly indicate that acridones show reducing ability (antioxidant activity) *in vitro*.

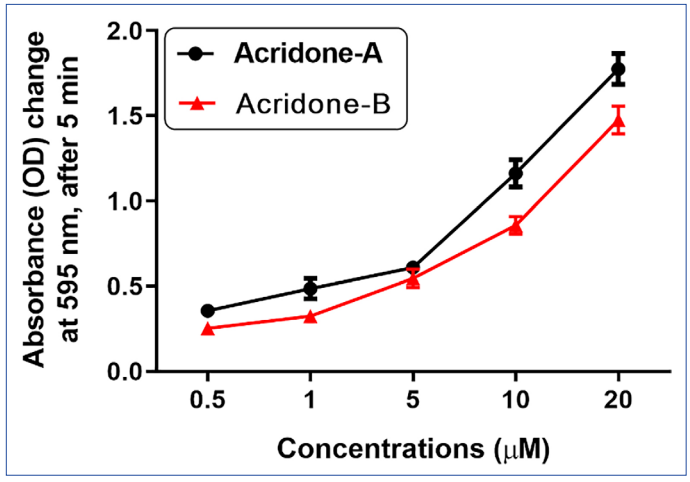
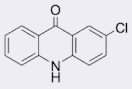
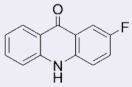


Figure 5. Concentration-dependent ferric reducing antioxidant properties of acridones.

Table 1. Absorbance change for 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals and ferric-reducing antioxidant power (FRAP) values of acridones at 65 min

Compounds	DPPH change of absorbance (ΔA_{517})	FRAP value (μM) Mean±SD
 A	0.89±0.021	34.27±2.9
 B	0.755±0.023	33.51±2.3

SD: Standard deviation

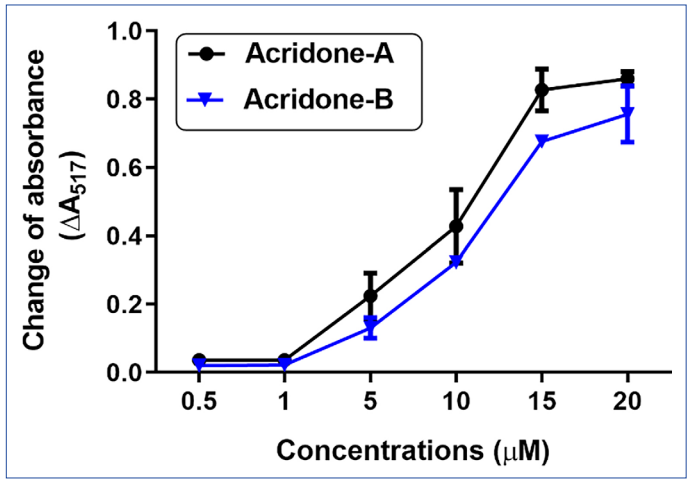


Figure 6. DPPH free radical-scavenging activity of acridones at various concentrations.

DPPH: 2,2-diphenyl-1-picrylhydrazyl.

Free radical scavenging activity *in vitro* DPPH assay

The free radical scavenging property of acridones was investigated using DPPH as a stable-free radical. The antioxidant activity of various substances is commonly assessed using DPPH [35]. The concentration-dependent scavenging ability of acridones on the DPPH free radical was examined. This involved testing different concentrations of acridones and measuring the extent to which the DPPH free radical could be neutralized. Figure 6 provides insights into the dose-response relationship, enabling to determine the efficacy of acridones as free radical scavengers. Scavenging ability of the DPPH free radical increases as the concentration of acridones increases. Overall, this investigation provides valuable information about the free radical scavenging property of acridones and their potential as antioxidants. The Figure 6 and Table 1 display compelling evidence indicating that acridones possess a potent scavenging property, specifically antioxidant activity.

Discussion

To combat protein oxidation caused by free heme and iron, two new acridones called 2-chloroacridin-9(10H)-one (A) and 2-fluoroacridin-9(10H)-one (B) were synthesized. These acridones were designed to possess iron chelation and heme interaction properties, which are crucial for combating oxidative stress and preventing protein oxidation. Acridones are a class of heterocyclic organic compounds that can be obtained from natural sources or synthesized using the acridine framework. They possess a structure comprising two fused benzene rings, with a nitrogen atom at position 10 and a keto group at position 9 [36]. Acridones have various applications, including medical use as topical antiseptics and in drug formulation [23, 36, 37]. They also exhibit antimicrobial properties and have been investigated as DNA intercalators [36, 38, 39]. The acridone scaffold was chosen for this experiment due to its inherent iron chelating and heme-interacting properties [26, 40]. Acridones are known for their ability to bind to iron ions and interact with heme molecules, making them suitable candidates for preventing protein oxidation caused by free heme and iron.

The synthesized acridones, 2-chloroacridin-9(10H)-one, and 2-fluoroacridin-9(10H)-one, have distinct electronic, steric, and chemical characteristics due to the specific substitutions of chlorine and fluorine atoms. These substitutions differentiate them from other acridones and significantly affect their interactions with iron and heme molecules. The presence of chlorine and fluorine atoms alters the electron density within the acridone structure, influencing the strength of intermolecular interactions involved in iron chelation and heme binding.

The heme-interacting properties of the acridones were evaluated, demonstrating a strong interaction with heme molecules. The chlorine and fluorine atoms in the synthesized acridones facilitated effective binding and interaction with heme through hydrogen bonding and π-π stacking interactions. Acridones can stack with the porphyrin ring of heme due to their planar aromatic structure and form stable complexes [41]. These interactions allow acridones to control heme availability, prevent heme oxidation, and reduce oxidative stress caused by heme release. Further research is required to fully understand the mechanisms underlying heme interaction.

The iron-chelating ability of the acridones was also evaluated, showing a strong capacity to chelate iron. The tricyclic structure of acridones provides functional groups, such as oxygen and nitrogen atoms, that act as donor sites for coordination with iron. Acridones effectively sequester iron ions by chelating them, preventing their contribution to damaging processes such as fenton chemistry [42]. This iron chelation action helps restore iron homeostasis and reduce oxidative stress [43]. Acridones show potential as iron chelators for the treatment of neurodegenerative diseases associated with iron dysregulation [44]. Additional investigation is required to comprehensively grasp the mechanisms of iron chelation by acridones and evaluate their safety and therapeutic efficacy in particular disease contexts.

The acridones were evaluated for their impact on heme- and iron-mediated protein carbonyl formation, a marker for oxidative stress [45]. The results indicated that the acridones can mitigate oxidative stress and protect proteins from damage induced by heme and iron. These findings suggest that acridones have potential as therapeutic interventions for heme-related disorders and oxidative stress-related illnesses. Furthermore, the acridones exhibited significant antioxidant activity, indicating their effectiveness as antioxidants. Acridones protect against oxidative stress caused by free iron and heme by scavenging reactive oxidants and free radicals generated by iron and heme-mediated oxidative processes. Their antioxidant action stabilizes reactive oxidants and prevents the chain reaction of oxidation [46]. This antioxidant activity is particularly relevant in the context of heme and iron, as they can catalyze the generation of reactive oxidants. Acridones alleviate oxidative stress and reduce cellular damage caused by heme and iron by scavenging and neutralizing reactive oxidants. They have potential therapeutic uses in oxidative stress-related conditions, such as cancer, cardiovascular disease, and neurological diseases. Additional research is necessary to gain a comprehensive understanding of the antioxidant mechanisms exhibited by acridones and evaluate their potential in therapeutic applications against cancer, cardiovascular disease, and neurological diseases.

In summary, the synthesis of novel acridone derivatives with iron chelation and heme interaction properties has significant implications for therapeutic interventions and biomarker development in oxidative stress-related disorders [47–49]. These derivatives have the potential to prevent free heme- and iron-mediated protein oxidation, reduce oxidative stress, and contribute to improved treatments and diagnostic tools. By understanding the role of oxidative stress in various diseases [50], advancements in patient care and outcomes can be achieved.

Conclusion

The article's conclusion highlights the effective synthesis of two new acridone with iron-chelating and heme-interacting characteristics. Since free heme and iron-mediated protein oxidation are prominent indicators of oxidative stress, these compounds have the potential to counteract it. The derivatives successfully lower the production of reactive oxidants and lessen protein oxidative damage by chelating iron and stabilizing heme molecules. These acridones may be used therapeutically to treat oxidative stress-related diseases, according to this study. To completely comprehend the mechanisms of action, enhance their efficacy, and determine the safety of using them in clinical settings, more study is necessary. The research presented in this article, taken as a whole, advances our knowledge of how new drugs might be developed to address the oxidative stress brought on by iron and heme dysregulation.

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