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**N'-(2,6-dimethoxybenzylidene)-3-(4-methoxyphenyl) acrylohydrazide abates oxidative stress and purinergic enzymes abnormality associated with iron-induced cardiotoxicity in Wistar rats: Experimental and computational perspectives**

Ogunlakin, Daniel Akingbolabo<sup>1,2,\*</sup>, Oluwafemi Adeleke Ojo<sup>1,2,\*</sup>, Evbuomwan Ikponmwosa<sup>3</sup>, Olaoluwadotun Dorcas Olanrewaju<sup>1,2</sup>, Adesewa Kehinde Ajiboye<sup>1,2</sup>, Damilare Iyinkristi Ayokunle<sup>4</sup>, Mubo Adeola Sonibare<sup>5</sup>, Abel Kolawole Oyebamiji<sup>6</sup>, Omolola Adenike Ajayi-Odoko<sup>7</sup>, Matthew Akin Ogunlakin<sup>8</sup>, Kevwe Benefit Esievo<sup>9</sup>

<sup>1</sup>Bowen University SDG 03 (Good Health and Wellbeing Research Cluster), Nigeria.

<sup>2</sup>Phytomedicine, Molecular Toxicology, and Computational Biochemistry Research Laboratory (PMTCB-RL), Department of Biochemistry, Bowen University, Iwo, 232101, Nigeria.

<sup>3</sup>Department of Microbiology, Landmark University, Omu-Aran, Nigeria.

<sup>4</sup>Department of Pure and Applied Biology, Bowen University, Iwo, Nigeria.

<sup>5</sup>Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan, Ibadan. Nigeria.

<sup>6</sup>Department of Chemistry and Industrial Chemistry, Bowen University, Iwo, Osun State, Nigeria.

<sup>7</sup>Microbiology programme, Bowen University, Iwo, 232101, Nigeria.

<sup>8</sup>Nestle Nigeria Ltd, Ilupeju. Lagos. Nigeria.

<sup>9</sup>Department of Medicinal Plant research and Traditional Medicine, National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria.

**Abstract**

The effects of N'-(2,6-dimethoxybenzylidene)-3-(4-methoxyphenyl) acrylohydrazide (KAD 9) on iron-induced cardiac injury was investigated. Evaluations of the iron chelating capability, ferric-reducing antioxidant power (FRAP), as well as DPPH free radical scavenging activity of KAD 9 were performed. The oxidative cardiac injury induced by 0.1 mM FeSO<sub>4</sub> was treated with varied doses of KAD 9 ex vivo. Comparing KAD 9 to conventional quercetin, the DPPH radical scavenging ability of KAD 9 significantly rises with concentration (p<0.05). The cardiac injury was generated, and this resulted in decreased malondialdehyde (MDA), catalase (CAT), ATPase, and ENTPDase activity (p<0.05). A notable increase in GSH level was seen in KAD 9 treated group. Furthermore, it was observed that KAD 9 had closer binding affinity with ATPase and ENTPDase. Due to its ability to regulate nucleotide hydrolysis and lessen oxidative stress, KAD 9 has the potential to both treat and protect against oxidative cardiac injury.

**Keywords:** N'-(2,6-dimethoxybenzylidene)-3-(4-methoxyphenyl) acrylohydrazide (KAD 9); iron-induced cardiac injury; antioxidant; ATPase; ENTPDase

\*Corresponding author: [gbolaogunlakin@gmail.com](mailto:gbolaogunlakin@gmail.com); +2347037883049

## Introduction

All living cells engage in aerobic metabolic processes, which culminate in the production of free radicals. Among the oxygen species produced by incomplete oxidation or decrease of oxygen are hydrogen peroxide ( $H_2O_2$ ), superoxide anion ( $O^{2-}$ ), and hydroxyl radical ( $HO\cdot$ ) [1,2]. The term "oxidative stress" is used to indicate the disproportionate generation and buildup of reactive oxygen species (ROS) in the tissues and cells, as well as the inability of a biological system to get rid of these reactive products using the system's antioxidant defense mechanism, as described by Debnath et al. [3], Sachdev et al. [4], and Ogunlakin et al. [5].

Cardiotoxicity is linked to a compromised cardiac endogenous antioxidant system, which causes the formation of ROS and oxidative stress within the heart and circulatory system. Additionally, the Fenton and Haber Weiss reactions, which iron catalyzes, substantially aggravate this state in the presence of iron [6]. Cardiotoxicity is a serious medical condition that, if not treated and managed properly and promptly, can result in heart failure, damage to the myocardial muscles, disruption of the heart's rhythmic activity, and even death of the person suffering from it [7-9]. Iron chelators like deferiprone, deferoxamine, and deferasirox are part of the conventional remedy for cardiotoxicity. Despite their drawbacks and high cost, these drugs are administered as treatment as well as in preventing cardiac iron overload as well as myocardial dysfunction. Increased

evidence from many studies that have focused on antioxidant pathways as an alternative therapeutic option has further confirmed the contribution of oxidative stress to cardiotoxicity. These findings are important because treating cardiotoxicity with iron chelating therapies could be unaffordable and have side effects on the victim [6, 10].

Research has shown that the compound cinnamic acid as well as its derivatives are effective and have antioxidant qualities [11]. Fruits as well as vegetables are the recognized sources of antioxidants such as cinnamic acid as well as its analogues. These compounds possess significant health advantages, including hepatoprotective [12], anticancer [13,14], anti-diabetic [15], as well as cardioprotective [6] activities. Recent research has focused on its ability to reduce blood glucose levels [16,17]. Recently, N'-(2,6-dimethoxybenzylidene)-3-(4-methoxyphenyl) acrylohydrazide (KAD 9) was reported to inhibit the proliferation of cervical adenocarcinoma (HeLa) and Chinese Hamster Ovarian (CHO-1) cells via complexing with p53 enzyme [5]. The goal of this investigation was to determine the benefits and therapeutic potential of a cinnamic acid analogue to prevent iron-induced cardiotoxicity. This is due to a lack of scientific data about the benefits of phytochemicals for cardiotoxicity, the drawbacks of iron-chelating drugs, and the difficulties people with cardiotoxicity encounter with their health. Therefore, in this study, N'-(2,6-

dimethoxybenzylidene)-3-(4-methoxyphenyl) acryloylhydrazide (KAD 9), a cinnamic acid analogue, was assessed for its antioxidant influence in iron-induced cardiac injury.

## Materials and Methods

### Materials and reagents chemicals

In a previous study [18], cinnamic acid (CAS 140-10-3; Merck, Germany) was derivatized to yield KAD 9, and quercetin (CAS 117-39-5) was acquired from Santa Cruz Biotechnology, Heidelberg, Germany. All compounds were of analytical grade. All absorbances were measured using a spectrophotometer (Spectra Max Plus, Molecular Devices, CA, USA).

### *In vitro* Antioxidant Activity

The conventional method [19] was used to measure KAD 9's iron chelating activity, while the method described by Ruan et al. [20] was used to measure its antioxidant potential via DPPH. Benzie and Strain's [21] procedures were followed to conduct the ferric-reducing antioxidant power (FRAP) experiment.

### *Ex vivo* studies

#### Experimental rats and organ preparation

From the Animal House at Bowen University in Iwo, Nigeria, healthy male Wistar rats (10–12 weeks old) that weighed 250–300 g each were

acquired. All animals were housed in cages with a 12-hour light/dark cycle at ambient temperature (20–25 °C). To absorb animal excrement, bedding made of softwood shavings was used within the cages, and it was changed frequently. They received free access to water and continual feeding pellets (supplied by Ladokun Feeds Nig. Ltd.) during this investigation. Animals were acclimated for at least one week before the start of the experimental procedures. The activity was carried out following the recommendations and the Guide for the Care and Use of Laboratory Animals. The use and care of animals was by all applicable local, institutional, national, and/or international laws and regulations. Additionally, the experiment received approval from Bowen University's institutional animal ethics committee (BUI/BCH/2022/0002). After fasting the previous night, ten rats were euthanized with halothane (Slott et al., 1994), and their hearts were then removed and homogenized in 1% Triton X-100 in 50 mM phosphate buffer. The homogenate was centrifuged at 15,000 rpm at a temperature of 4 °C. For *ex vivo* investigation, the supernatants were collected in simple tubes and kept at -40 °C.

#### Cardiac injury induction

With a few minor alterations, the procedure described by Ojo et al. [12] was used. 200 µL of the organ supernatant was mixed with 100 µL of 0.1 mM FeSO<sub>4</sub> and various KAD 9 concentrations (30, 60, 120, and 240 g/mL). After being incubated for 30 minutes at 37 °C, the

mixture was used for biochemical analyses. Reaction mixture with only the organ supernatant was used as the positive control, whereas reaction mixtures with only the tissue supernatant and FeSO<sub>4</sub> were utilized as the negative control.

#### Measurement of antioxidant activities

##### Level of glutathione (GSH)

According to Salau et al. [6], 10% trichloroacetic acid was used to deproteinize 600 mL of the tissue lysates. After that, the mixture was centrifuged at 3500 rpm for 10 minutes. 500 mL of the sample and 100 mL of the Ellman reagent were added to a clean test tube. At 415 nm, the absorbance was determined during a 5-minute incubation period at 25 °C. The GSH was utilized as a guide.

##### Catalase (CAT) activity

The technique outlined by Ojo et al. was used to analyze the CAT activity for KAD 9 with a little modification [12]. 20 mL of tissue samples contained several doses of KAD 9, and 780 µL of 50 mM phosphate buffer was added to those samples. Therefore, after adding 300 L of 2M H<sub>2</sub>O<sub>2</sub>, the absorbance was measured at 240 nm for 3 minutes at intervals of one minute.

##### Level of lipid peroxidation

KAD 9's ability to prevent lipid peroxidation was evaluated using the procedure outlined by Ojo et al. [12]. 100 µL of tissue lysates with variable concentrations of KAD 9 were introduced

progressively to 375 µL of 20% acetic acid, 1000 µL of 0.25% thiobarbituric acid, and 100 µL of 8.1% SDS. The reaction mixture was warmed up for 60 min at 95 °C (in a water bath). After the mixture had cooled to room temperature, the absorbance at 532 nm was measured.

##### Purinergic activity

Na<sup>+</sup>/K<sup>+</sup> ATPase and E-NTPDase enzymes activity

The method as reported by Erukainure et al. (2017) was slightly adjusted to test the Na<sup>+</sup>/K<sup>+</sup> ATPase activity. The absorbance was calculated at 660 nm. The Ojo et al. [12] procedure was used, with a few minor changes, to determine the E-NTPDase enzyme activity. The absorbance at 600 nm was measured after a 10-minutes incubation period at -4 °C.

##### Molecular Docking Study

The docking study was executed using molecular operating environment software 2022 version. Appropriate atoms were bonded together to form KAD 9 and the modelled compound was optimized before saving it in .moe format. The studied receptors (ATPase (PDBID: 6j17), and ENTPDase (PDB ID: 3ZX3) [22,23] were retrieved from protein data bank and were treated by removing the water molecules as well as the native ligand structures before they were optimized for further study. The active site(s) were located and the default site was selected for the docking investigation. Induce fit approach

was employed for the study and the obtained result were examined and reported appropriately.

### Data analysis

Software called Graphpad Prism 9.0.1 was used to analyze the data. The descriptive data were represented by the mean standard deviation ( $\pm$ SD). One-way ANOVA and Tukey's post hoc analysis, at a significance level of  $p < 0.05$  was used to compare the mean.

### Results

#### Antioxidant assays

The in vitro antioxidant ability of KAD 9 was evaluated as mean percentage inhibition and contrasted with that of quercetin, the reference antioxidant. Figures 1-3 show how the KAD 9's capacity to scavenge DPPH radicals, reduce ferric ions ( $\text{Fe}^{3+}$ ), and chelate ferrous ions ( $\text{Fe}^{2+}$ ) at various concentrations. KAD 9 did, however, scavenge DPPH radicals, especially as concentration increased, with the maximum scavenging activity of the substance found at 240  $\mu\text{g/mL}$  (Figure 1). When compared to the standard, the compound's radical scavenging activity was slightly lower. Even at the greatest concentration (240  $\mu\text{g/mL}$ ), the substance's ability to reduce ferric ( $\text{Fe}^{3+}$ ) was significantly lower (Figure 2). Again, the compound's ability to reduce antioxidant activity was unaffected by concentration. Using quercetin as a reference, Figure 3 reports the percentage of inhibition of KAD 9's  $\text{Fe}^{2+}$ -chelating activity using quercetin

as the standard. KAD 9, as depicted in Figure 3, exhibited notable ferrous ( $\text{Fe}^{2+}$ ) ion chelation ability in a dose-independent manner, meaning that the chelation ability of the compound was unaffected as concentration was increased. Though equivalent performance between KAD 9 and the standard was seen at the lowest concentration, it was also low in comparison to the standard.

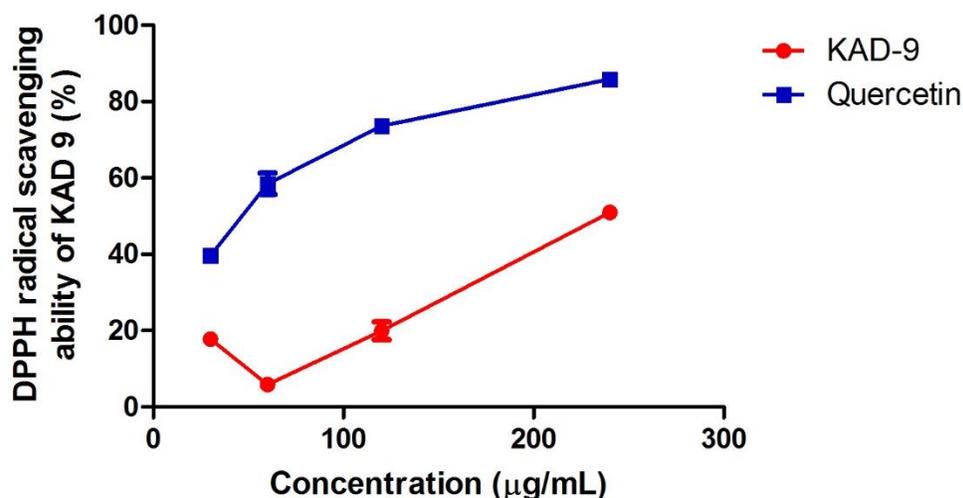
#### Ex vivo study

As seen in Figures 4-6, treatment with KAD 9 in  $\text{FeSO}_4$ -induced oxidative cardiac injury in rat hearts resulted in a significant ( $p < 0.05$ ) depletion in CAT and GSH levels, and on the other hand, a concurrent increase in MDA level, indicating the presence of oxidative stress on the treatment of rat cardiac tissues with  $\text{FeSO}_4$ . At varied doses of KAD 9 the enzyme activities were significantly ( $p < 0.05$ ) reversed, and the levels of GSH, CAT, and MDA were nearly normal. Treatment with KAD 9, as shown in Figure 4, reverted the levels of the GSH activities to nearly normal levels. This effect was dose-dependent. The highest dose (240  $\text{mg/mL}$ ) had the most activity. Additionally, the results for CAT demonstrated a dose-dependent reversal of the enzyme's activity from 60 to 240  $\text{mg/mL}$  that was significant ( $p < 0.05$ ) (Figure 5). But at the maximum dose (240  $\text{mg/mL}$ ), a significant improvement in KAD 9's activity was observed when compared to the negative control (tissue that had not been treated with KAD 9). Surprisingly, the lowest dose of KAD 9 (30  $\text{mg/mL}$ ) showed the maximum activity, much

higher than the negative control (tissue that had not been treated). The highest amounts of MDA were found at 30 and 240 mg/mL, respectively (Figure 6), although this increase was dose-independent. Figures 7 and 8 show the investigation of the impact of aqueous KAD 9 on the ATPase and ENTPDase activities in iron-mediated oxidative cardiac damage. After the generation of heart damage, there was a considerable increase in cardiac ATPase activity (Figure 7,  $p < 0.05$ ), which was accompanied by a decrease in cardiac ENTPDase activity (Figure 8). A dose-dependent increase in cardiac ENTPDase activity was accompanied by a decrease in ATPase activity after treatment with KAD 9, which was significantly ( $p < 0.05$ ) proved to have an inhibitory impact.

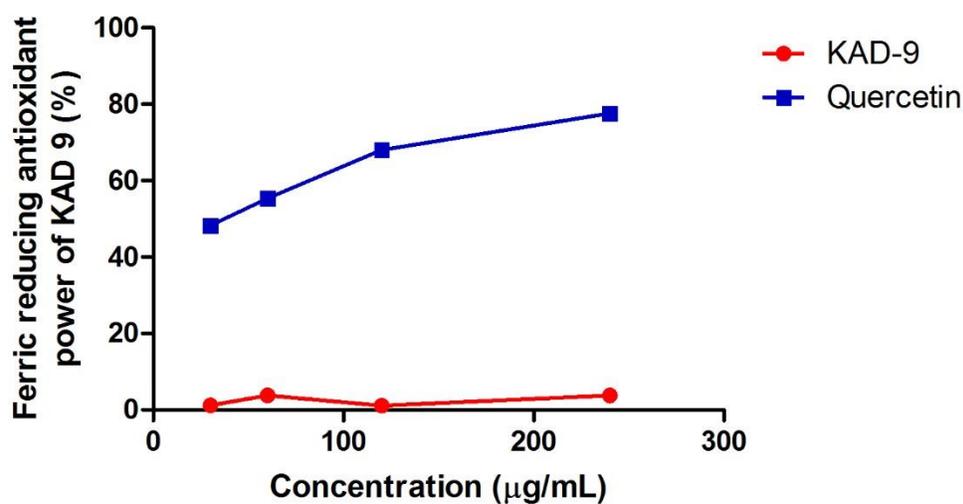
### Molecular docking

As shown in table 1, the calculated scoring revealed the potential inhibiting capacity of N'-(2,6-dimethoxybenzylidene)-3-(4-methoxyphenyl) acrylohydrazide (KAD 9) against ATPase and ENTPDase. The calculated binding affinities for KAD 9 against ATPase and ENTPDase were  $-7.10992289$  kcal/mol and  $-5.75579119$  kcal/mol respectively. The obtained results for the studied complexes were compared against docked quercetin against the studied receptors and were reported in table 1. More so, the amino acid residues involved in the interaction were ARG 1110, ARG 1110, THR 1114 for ATPase and ARG 303, LYS 330 for ENTPase (Figures 9 - 12).



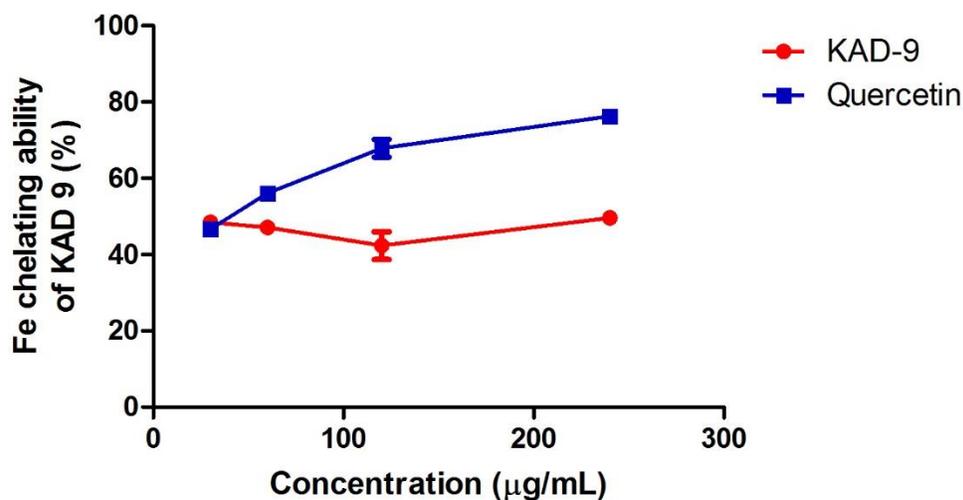
**Figure 1:** DPPH scavenging ability of KAD 9

Data expressed as mean  $\pm$  SD (n = 3).



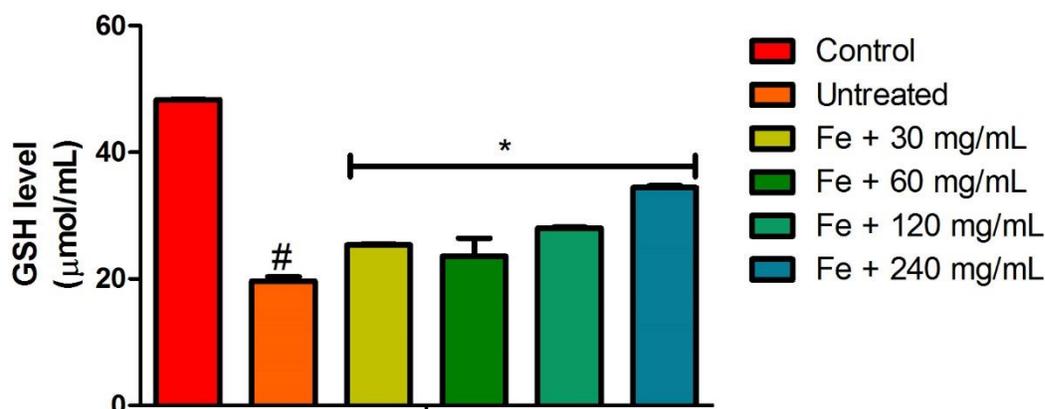
**Figure 2:** Ferric reducing antioxidant power of KAD 9

Data expressed as mean  $\pm$  SD (n = 3).



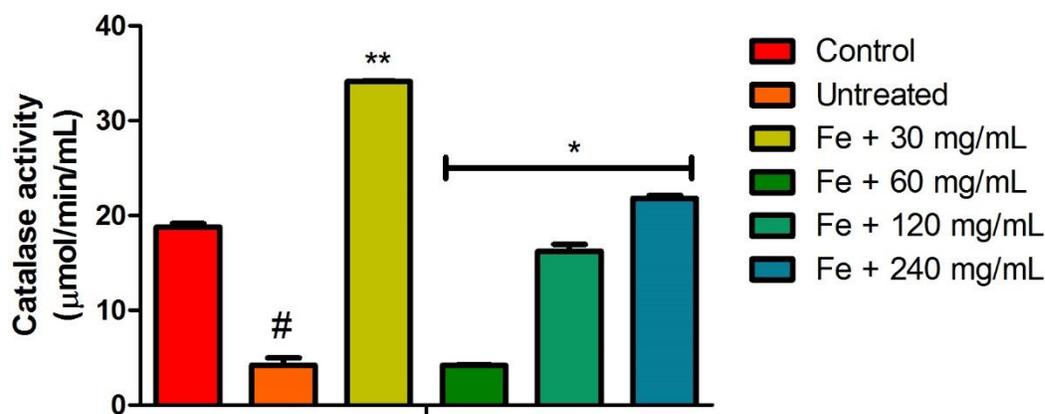
**Figure 3:** Iron chelating ability of KAD 9

Data expressed as mean  $\pm$  SD (n = 3).



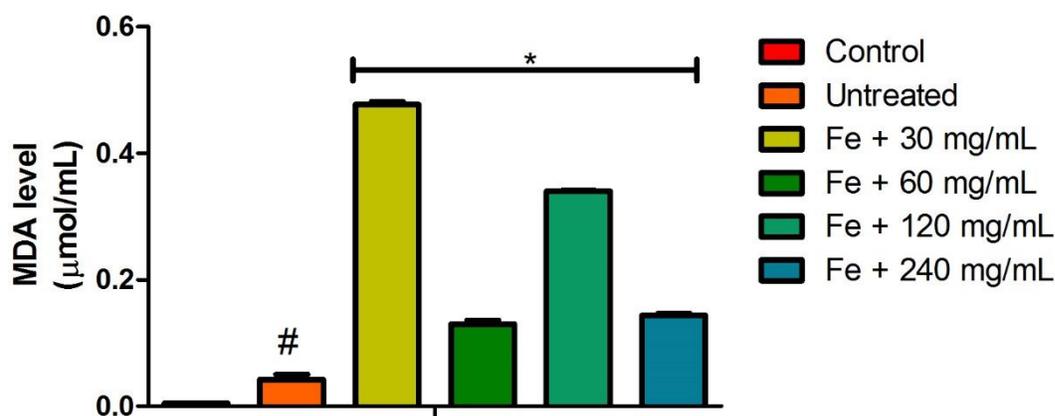
**Figure 4:** Effect of KAD 9 on GSH level in iron-mediated oxidative cardiac damage.

Data: mean SD; n = 3. \* = statistically significant difference from untreated tissue; # = statistically significant difference from normal tissue.



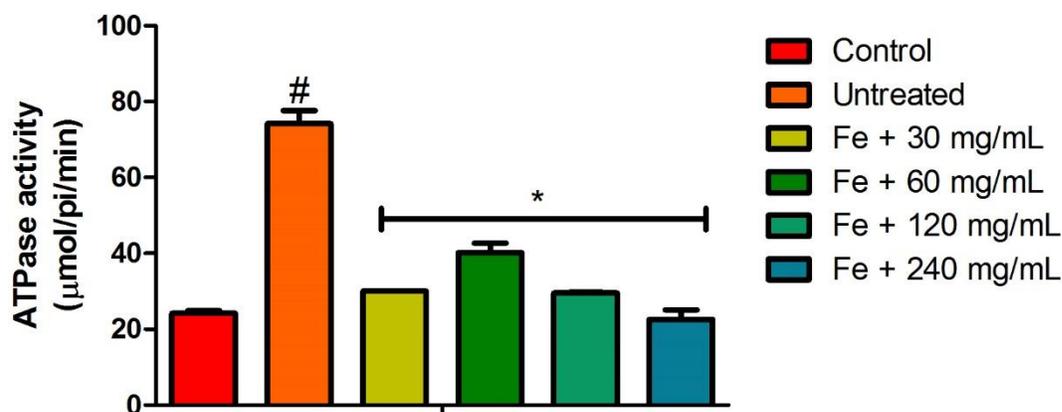
**Figure 5:** Impact of KAD 9 on catalase activity in iron-mediated oxidative heart injury.

Data are presented as mean±SD with a sample size of 3. Data: mean SD; n = 3. \* = statistically significant difference from untreated tissue; # = statistically significant difference from normal tissue.



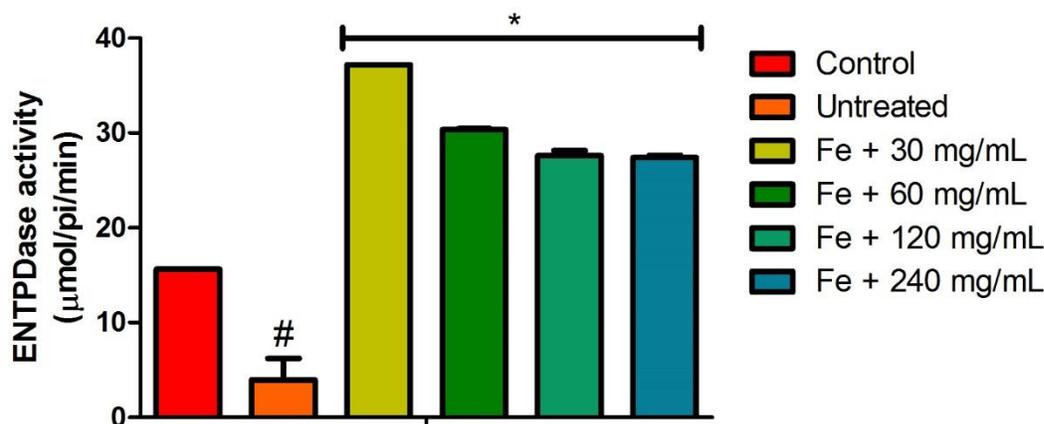
**Figure 6:** Effect of KAD 9 on the amount of MDA in iron-mediated oxidative cardiac injury.

Data: mean±SD; n = 3. Data: mean SD; n = 3. \* = statistically significant difference from untreated tissue; # = statistically significant difference from normal tissue.



**Figure 7:** KAD 9's impact on ATPase function in iron-mediated oxidative cardiac damage.

Data: mean±SD; n = 3. Data: mean SD; n = 3. \* = statistically significant difference from untreated tissue; # = statistically significant difference from normal tissue.

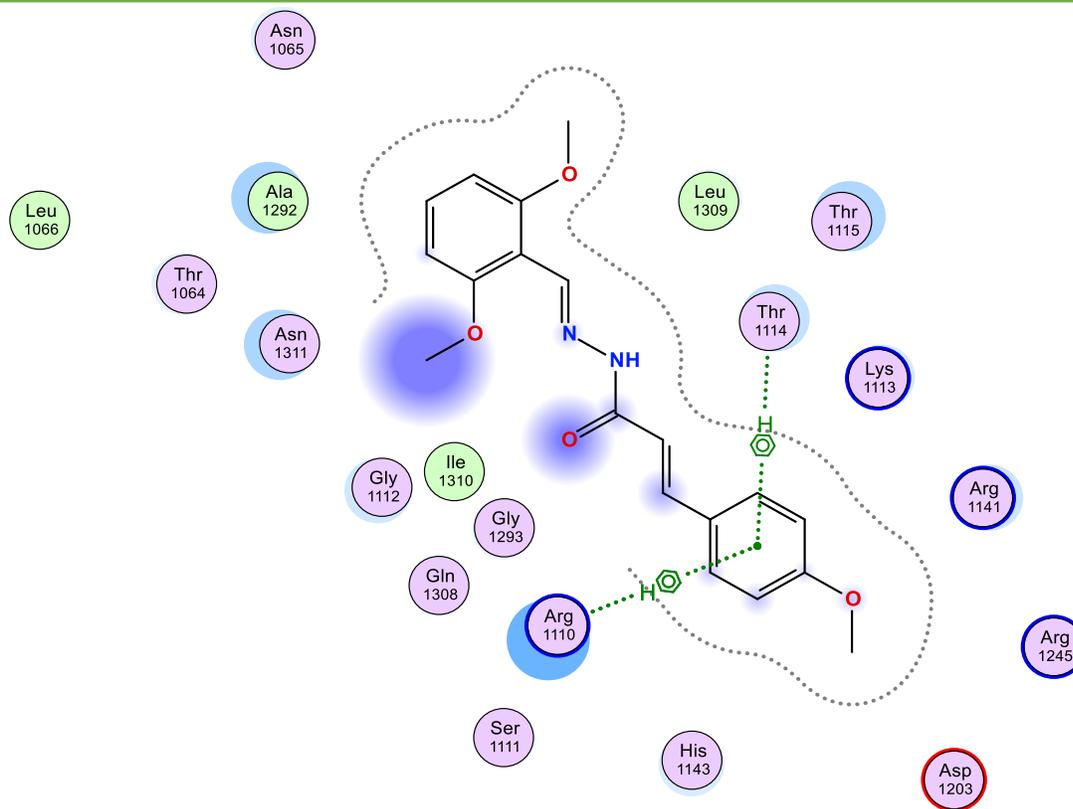


**Figure 8:** KAD 9 impact on ENTPDase activity in iron-mediated oxidative cardiac injury.

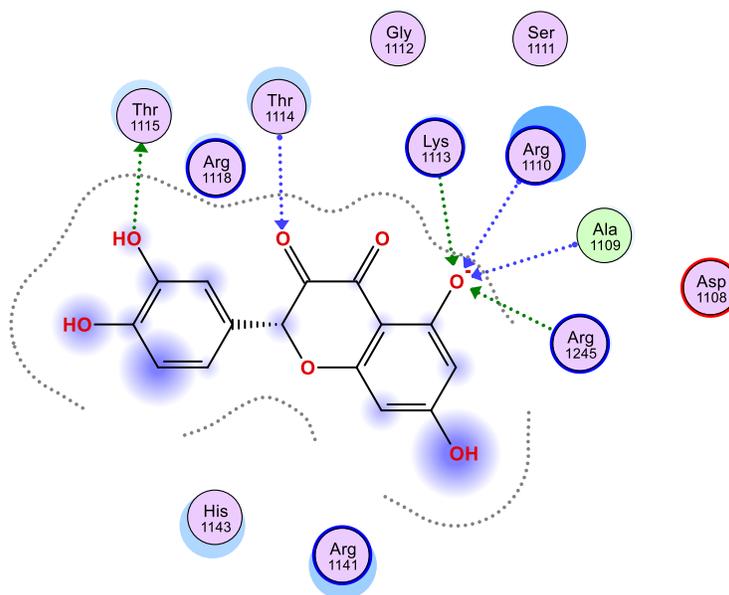
Data: mean±SD; n = 3. Data: mean SD; n = 3. \* = statistically significant difference from untreated tissue; # = statistically significant difference from normal tissue.

**Table 1:** Calculated binding affinity for the studied complexes

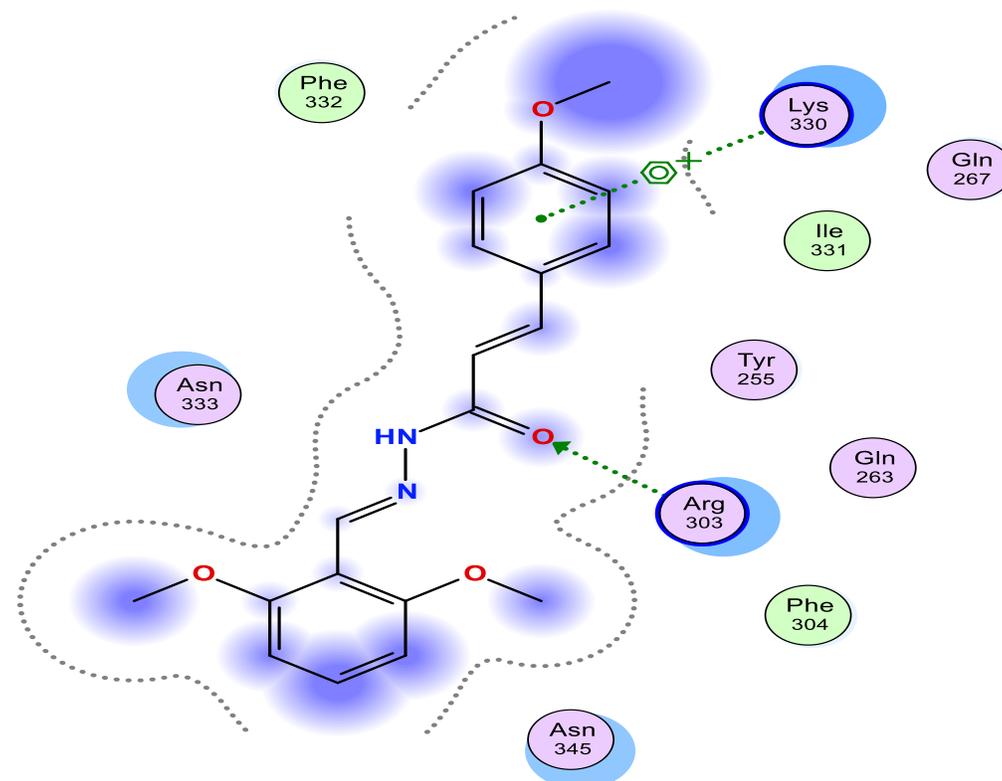
	ATPASE		ENTPDASE	
	Binding Affinity (kcal/mol)	Amino Acid Residue	Binding Affinity (kcal/mol)	Amino Acid Residue
KAD 9	-7.10992289	ARG 1110, THR 1114	-5.75579119	ARG 303, LYS 330
Quercetin (Reference)	-7.33278513	THR 1115, THR 1114, ALA 1109, ARG 1110, ARG 1245, LYS 1113	-6.27352333	GLY 187, LYS 190



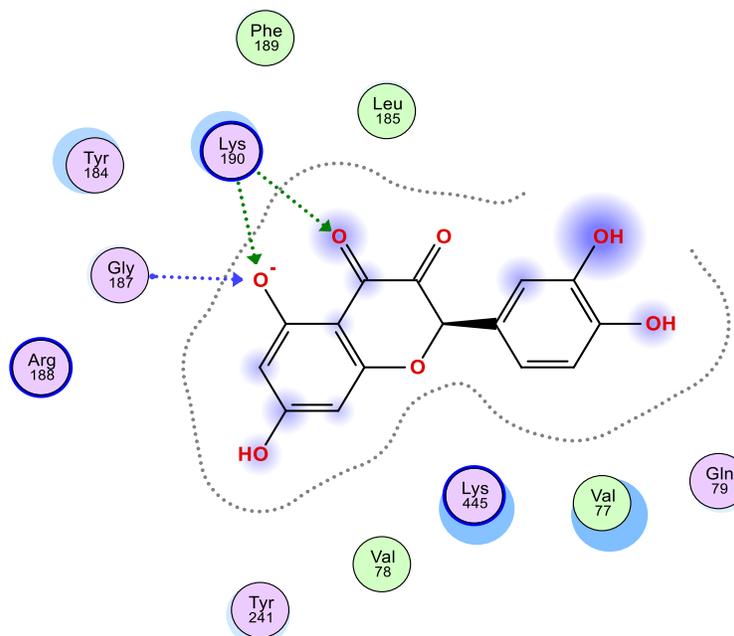
**Figure 9:** 2D structure of KAD9 against ATPase



**Figure 10:** 2D structure of quercetin against ATPase.



**Figure 11:** 2D structure of KAD9 against ENTPDase



**Figure 12:** 2D structure of quercetin against ENTPDase

## Discussion

The results of antioxidant activities of KAD 9 evaluated through DPPH radical scavenging ability,  $\text{Fe}^{3+}$  reducing antioxidant power, and  $\text{Fe}^{2+}$  chelation ability showed the direct association between the compound and antioxidant activity. In this study, KAD 9 effectively scavenged DPPH in a dose-dependent mode but did not efficiently reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  *in vitro*. The aptitude to hunt DPPH free radical could be linked to the phenolic content of the compound. Similar observation has been reported in which DPPH scavenging capacity was linked to the amount of phenolics present [24]. By the Fenton and Haber-Weiss reactions, in which ferrous ions ( $\text{Fe}^{2+}$ ) combine with  $\text{H}_2\text{O}_2$  to produce the highly reactive  $\cdot\text{OH}$ , ferrous ions ( $\text{Fe}^{2+}$ ) contribute to the creation of ROS, which can catalyze lipid peroxidation [25]. This  $\cdot\text{OH}$  can have a negative impact on important macromolecules such as proteins, nucleic acids, as well as lipid. This reducing power assay can be evaluated based on the intensity as well as the absorbance of the reddish colour reduced  $\text{Fe}^{2+}$  in the  $\text{Fe}(\text{II})(\text{FZ})_3$  complex [26]. Hence, chelating potential is regarded as an important measure of antioxidant activity. In this assay, KAD 9 efficiently chelates  $\text{Fe}^{2+}$  dose-dependently. This chelating ability could be linked to the phenolic content of the compound. The potential of KAD 9, a phenolic compound, to chelate powerful prooxidant metal ions including iron lends credence to the antioxidant potential of KAD 9.

The chelating ability of KAD 9 could be linked to the hydroxyl group in the compound. The phenolic structure of phenolic compounds, coupled with the number as well as distribution of the  $\text{OH}$  groups, determine how effective they are at chelating metals [27]. Hydroxyl groups in the benzene ring of cinnamic acid derivatives are required for  $\text{Fe}^{2+}$  chelation [28]. As demonstrated in this study, KAD 9 has antioxidant and chelating properties, which may play a crucial role in preventing oxidative damage caused by metal-catalyzed decomposition reactions [29,30].

Cardiotoxicity is a significant health concern that can cause damage to the cardiac muscles and impair the heart healthy function, a condition, if not adequately treated and managed, can lead to cardiac failure [31]. This condition can result from exposure to several substances including chemotherapy drugs, certain medications, alcohol, as well as environmental toxins [32-35]. Deteriorated bioenergetics, lipotoxicity, and oxidative imbalance have also been connected to the cardiovascular illness's pathogenesis [36,37]. Natural products have become more popular as an alternative source of readily available, reasonably priced treatments with few to no negative side effects due to the high expense of therapy and the probable side effects of synthetic medications. In ferrous sulphate-induced cardiotoxicity, the current investigation shows that KAD 9 has cardioprotective effects. Cardiotoxicity and other heart-related dysfunctions have been linked to oxidative stress as a key pathogenic mechanism

[38-40]. Following the production of oxidative cardiac toxicity, the levels of GSH as well as catalase activity were depleted after incubation with FeSO<sub>4</sub>, which is suggestive of oxidative damage. The elevated amount of cardiac MDA, which denotes a peroxidative impact on the cardiac lipids following the development of oxidative heart damage, supports this even further. These changed activities and levels of these oxidative markers are in line with past studies on the occurrence of oxidative stress after inducing cardiac oxidative damage [36, 41-43]. By accelerating the Fenton's and Haber-Weiss reactions, which increase superoxide (O<sup>2-</sup>) generation, iron toxicity, leads to oxidative stress [44].

KAD 9 treatment of cardiac tissues resulted in increased GSH levels and catalase activity, which point to the compound's antioxidative properties. This supports earlier studies that found that plants' compounds possess antioxidant cardioprotective properties [36, 42-45]. The increasing amount of MDA, however, indicated that KAD 9 did not exhibit anti-peroxidative activity. Adenosine, an endogenous signaling nucleotide that can widen coronary arteries, is crucial for maintaining the heart's regular physiological processes [9,46]. Additionally, adenosine has been demonstrated to mitigate catecholamines' detrimental effects on the myocardium [47]. Cardiac ATPase activity increases and ENTPDase activity decreases in response to oxidative stress, indicating a decrease in the levels of both ATP and adenosine.

According to past research on altered purinergic enzyme activities in oxidative damage [36, 48], the levels of enzyme activity in untreated cardiac tissues are consistent. Since ATP is an essential energy-signaling molecule, the increased ATPase activity denotes a change in bioenergetic activity and points to a lower myocardial ATP level. According to Das and Maulik [49], changes in cardiac bioenergetics have been linked to the pathophysiology of cardiovascular dysfunctions like cardiotoxicity, oxidative stress, and other cardiovascular system dysfunctions. According to research by Kirby et al. [50], ATP and adenosine both have vasodilatory effects on coronary arteries. Following treatment with KAD 9, the enzyme ATPase's activity decreased whereas ENTPDase's activity increased, indicating higher purinergic activity, which could indicate improved bioenergetics.

In this work, the activity of atoms presents in the studied ligands against the amino acid residues present in the studied receptors were investigated using molecular operating environment (MOE) software. Adeoye *et al.* [51] reported that the lower the binding affinity value, the better the ability of the studied ligand to inhibit the target and the steadier the interaction; thus, the calculated binding affinity for KAD 9 against ATPase and ENTPDase showed that KAD 9-ATPase complex proved to be more stable than KAD 9 – ENTPDase complex. The steady interaction observed for KAD 9-ATPase complex could be attributed to Pi-interactions between the ring system of the Phenyl in KAD 9

and Thr 1114 as well as Arg 1110 which thereby agreed with experimental result shown above. More so, it was observed that KAD 9 had closer binding affinity to scoring obtained for Quercetin.

### Conclusion

Overall, this study's findings suggest that KAD 9 may have some use in preventing OS-related cardiac injury. KAD 9 can control nucleotide hydrolysis and decrease oxidative stress, which suggests that it may be able to guard against oxidative cardiac injury. KAD 9 may therefore be an appropriate and powerful modality to help treat cardiac injury.

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