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ARIF ALI

BIOPROSPECTING HYDROXYLATED CHALCONES IN *IN VITRO* MODEL OF ISCHEMIA-REOXYGENATION AND PROBING NOX4 INTERACTIONS VIA MOLECULAR DOCKING

FORTALEZA

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Thesis presented to the Postgraduate Program in Pharmacology of the Faculty of Medicine of the Federal University of Ceará, as a partial requirement for obtaining the title of Doctorate in Pharmacology. Concentration area: Pharmacology.

Advisor: Profa. Dra. Alice Maria Costa Martins.
Co-advisor: Prof. Dr. Geraldo Bezerra da Silva Junior

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Approved on: 11/06/2024.

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Arif Ali

RESUMO

Bioprospecção de chalconas hidroxiladas em modelo *in vitro* de isquemia-reoxigenação e sondagem de interações NOX4 via docking molecular

A lesão de isquemia e reperfusão (I/R) é uma causa prevalente de lesão renal aguda (LRA) em várias condições clínicas, incluindo transplante renal, cirurgias cardíacas e nefrectomia, contribuindo significativamente para a alta taxa de mortalidade e morbidade em todo o mundo. O objetivo deste estudo foi avaliar o papel protetor das 2'-hidroxichalconas no tratamento da LRA induzida por I/R, visando as principais vias patológicas, incluindo geração de ROS, apoptose precoce, necrose, sistema antioxidante e interações NOX4. Os derivados das chalconas são amplamente estudados devido à sua estrutura simples e inúmeras atividades biológicas, como ações antioxidantes, anti-inflamatórias, antimicrobianas, anticancerígenas, antidiabéticas, psicoativas e neuroprotetoras. Portanto, considerando a forte ação antioxidante juntamente com outros papéis farmacológicos dos derivados da chalcona, seis 2'-hidroxichalconas foram sintetizadas por meio de uma reação de condensação de Claisen-Schmidt iniciada com 2-hidroxiacetofenona. Esses derivados de chalcona foram então analisados quanto ao seu papel protetor na LRA induzida por I/R em células HK-2. Primeiramente, a viabilidade celular foi analisada por meio do ensaio MTT, seguido de citometria de fluxo para apoptose, necrose, geração de ROS e avaliação do potencial transmembrana mitocondrial. O efeito das moléculas de chalcona no balanço oxidação-redução foi avaliado analisando seu efeito sobre TBARS, GSH e SOD. As alterações morfológicas induzidas pela I/R foram avaliadas por meio de microscópio eletrônico de varredura (MEV) e para identificar uma interação com NOX4, foi realizado docking molecular. Os resultados do estudo mostraram que, entre seis 2'-hidroxichalconas; (E)-1-(2-hidroxifenil)-3-(4-metoxifenil)-prop-2-en-1-ona (chalcona A4) aumentou significativamente a viabilidade das células HK-2 em comparação com o grupo I/R. A chalcona A4 reduziu os eventos de morte celular reduzindo a geração de ROS citoplasmáticas e o potencial transmembrana mitocondrial. Da mesma forma, o tratamento com chalcona A4 aumentou a atividade de GSH e SOD enquanto reduziu o nível de TBARS, sinalizando sua forte ação antioxidante. As imagens de MEV mostraram ação protetora da chalcona A4 em células HK-2, onde reverteu as alterações morfológicas induzidas por I/R, incluindo apoptose, bolhas e fragmentação do citoplasma. Além disso, o estudo in silico da chalcona A4 demonstrou potenciais interações com a enzima NADPH oxidase 4, fornecendo assim mais evidências de seu papel protetor contra a LRA induzida por I/R. Esses resultados mostraram que o chalcona A4 possui ação protetoras potenciais contra danos celulares induzidos por I/R, possivelmente devido ao seu forte potencial antioxidante e interação com a subunidade NOX4 da NADPH oxidase.

Palavras-chave: Estresse oxidativo; mitocôndria; apoptose; antioxidante.

Abstract

Bioprospecting hydroxylated chalcones in *in vitro* model of ischemia-reoxygenation and probing NOX4 interactions via molecular docking

Ischemia reperfusion injury (I/R) is a prevalent cause of acute kidney injury (AKI) in various clinical conditions including kidney transplant, cardiac surgeries, and nephrectomy, significantly contributing to high rate of mortality and morbidity around the globe. The aim of this study was to evaluate the protective role of 2'-hydroxychalcones in treatment of I/R induced AKI by targeting main pathological pathways including generation of ROS, early apoptosis, necrosis, antioxidant system and NOX4 interactions. Chalcones derivatives are studied widely due to their simple structure and numerous biological activities such as antioxidant, anti-inflammatory, anti-microbial, anticancer, antidiabetic, psychoactive and neuroprotective actions. Therefore, considering strong antioxidant action along with other pharmacological roles of chalcone derivatives, six 2'-hydroxychalcones were synthesized through a Claisen-Schmidt condensation reaction initiated with 2-hydroxyacetophenone. These chalcone derivatives were then analyzed for their protective role in I/R induced AKI in HK-2 cells. Primarily, cell viability was analyzed via MTT assay, followed by flow cytometry for apoptosis, necrosis, ROS generation and mitochondrial transmembrane potential evaluation. The effect of chalcone molecules on oxidation- reduction balance was evaluated by analyzing its effect on TBARS, GSH and SOD. The I/R induced morphological changes were assessed via scanning electron microscope (SEM) and to identify an interaction with NOX4, molecular docking was performed. The results of the study showed, that among six 2'-hydroxychalcones, (E)-1-(2-hydroxyphenyl)-3-(4-methoxyphenyl)-prop-2-en-1-one (chalcone A4) has significantly increased the HK-2 cells viability compared to I/R group. Chalcone A4 has reduced the cell death events by reducing generation of cytoplasmic ROS and mitochondrial transmembrane potential. Similarly, chalcone A4 treatment has increased GSH and SOD activity while reduced the level of TBARS, signaling towards its strong antioxidant action. The SEM images showed chalcone A4 protective action on HK-2 cells where it has reversed the I/R induced morphological alterations including apoptosis blebbing's and cytoplasm fragmentation. Moreover, *in silico* study of chalcone A4 demonstrated potential interactions with NADPH oxidase 4 enzyme, hence providing further evidences of their protective role against I/R induced AKI. These results showed that chalcone A4 possess potential protective action against I/R induced cellular damage possibly due to its strong antioxidant potential and interaction with NOX4 subunit of NADPH oxidase.

Keywords: Oxidative stress; mitochondria; apoptosis; antioxidant.

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LIST OF SYMBOLS AND ABBREVIATION

A1	(E)-3-(furan-2-yl)-1-(2-hydroxyphenyl) prop-2-en-1-one
A2	(E)-3-(4-chlorophenyl)-1-(2-hydroxyphenyl) prop-2-en-1-one
A3	(E)-1-(2-hydroxyphenyl)-3-phenylprop-2-en-1-one
A4	(E)-1-(2-hydroxyphenyl)-3-(4-methoxyphenyl) prop-2-en-1-one
A5	(2E,4E)-1-(2-hydroxyphenyl)-5-phenylpenta-2,4-dien-1-one
A6	(E)-3-(2-fluorophenyl)-1-(2-hydroxyphenyl) prop-2-en-1-one
ADQI	Acute dialysis quality initiative
AKI	Acute kidney injury
AKIN	Acute kidney injury network
AxPE	Annexin V-Phycoerythrin
CKD	Chronic kidney disease
DCFH-DA	2′-7′-dichlorodihydrofluorescein diacetate
ESRD	End stage renal disease
ETC	Electron transport chain
GFR	Glomerulus filtration rate
GPx	Glutathione peroxidase
GSH	Glutathione
I/R	Ischemic reperfusion injury
I/R	Ischemia and reoxygenation
ICU	Intensive care unit
MDA	Malondialdehyde
MPO	Myeloperoxidase
MPTP	Mitochondrial permeability transition pore
MTT	3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
Nrf 2	Nuclear factor erythroid 2-related factor-2
Nrf-2	Nuclear factor erythroid 2-related factor-2
PI	Propidium iodide
PI3K/Akt	Phosphatidylinositol-3-kinase
PS	Phosphatidylserine

Rho123	Rhodamine 123
ROS	Reactive oxygen species
RRT	Renal replacement therapy
SCr	Serum creatinine
SEM	Scanning electron microscope
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TLR	Toll like receptor
ΔΨm	Mitochondrial membrane potential

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1. Introduction

Acute kidney injury (AKI) is referred to rapid loss of kidney function characterized by increased level of serum creatinine and decreased urine output lasting for seven days (KHWAJA, 2012). AKI has been linked to notable extensions in hospital stays, increase costs on health care, and higher mortality rate among hospitalized patients. Despite the continued upward trend of mortality rates, there has been prominent decrease trend over the last decade, indicating extraordinary advances in patient care. This favorable trend can be due to a variety of causes, including advances in dialysis care, increased availability of drugs with lower nephrotoxic characteristics, and judicious use of dopamine and diuretics (SUSANTITAPHONG; CRUZ; CERDA; ABULFARAJ *et al.*, 2013). However, the improvements in AKI patients survival have also resulted into long-term ramifications including increased susceptibility to cardiovascular diseases hospital readmissions, recurrent AKI episodes, development of chronic kidney disease (CKD), and poor quality of life (GAMEIRO; MARQUES; LOPES, 2021).

The definition of AKI has evolved over the years as new pathological pathways and diagnostic criteria were identified. The first consensus definition of AKI was published by Acute Dialysis Quality Initiative (ADQI) based on a set of principles termed as RIFLE which includes five stages; Risk, Injury, Failure, Loss and End-stage renal disease (BELLOMO; RONCO; KELLUM; MEHTA *et al.*, 2004). The RIFLE criteria have categorized AKI into three classes based on its severity (Risk, Injury and Failure) while the last two stages (Loss and End-stage renal disease) are designated as outcome classes. According to RIFLE criteria the three classes of AKI are based on alteration in serum creatinine ≥50% from baseline level or fall in glomerular filtration rate ≥25% or decrease in urine output 0.5 ml/kg/h for 6 h (HOSTE; CLERMONT; KERSTEN; VENKATARAMAN *et al.*, 2006).

The RIFLE criteria have two main limitations, it is dependent on previous data of baseline serum creatinine level and there is no clear information regarding renal replacement therapy (RRT). Therefore, a new definition of AKI was introduced by Acute kidney injury network (AKIN), it identifies AKI from rise in serum creatinine ≥0.3 mg/dl within 48 hours duration instead of depending on baseline level. AKI has been divided into stages 1-3, much like the RIFLE method, with the requirement for RRT automatically placing a patient in stage 3 (JOANNIDIS; METNITZ; BAUER; SCHUSTERSCHITZ *et al.*, 2009; MEHTA; KELLUM; SHAH; MOLITORIS *et al.*, 2007).

The International Kidney Disease Improving Global Outcomes (KDIGOs) guideline have consolidated both RIFLE and AKIN criteria's for defining AKI in more harmonized mode. They have preserved the requirement of a minimum increase in serum creatinine level of ≥0.3 mg/dL within a 48-hour period, as per the AKIN definition and reverted to a 7-day timeframe for the criterion involving a ≥50% increase in serum creatinine level. Moreover, KDIGOs have also included those AKI patients which are not covered by RIFLE or AKIN criteria (KELLUM; LAMEIRE; ASPELIN; BARSOUM *et al.*, 2012; PALEVSKY; LIU; BROPHY; CHAWLA *et al.*, 2013).

AKI pose significant burden on global healthcare system. Its prevalence and incidences vary across regions, populations and patient type, which may reflect differences in healthcare infrastructure and risk factors distribution. For instance, in numerous healthcare systems of high-income countries there is growing availability of kidney function either in electronic form, data link or other form of health record. These progressions have led to the acknowledgment that even small alterations in creatinine level results in negative prognosis (SAWHNEY; FRASER, 2017). However, AKI is recognized a worldwide health concern having significant mortality and morbidity rate (TEO; LEE; KONIMAN; TNG *et al.*, 2019).

Globally, AKI-related mortality exceeds that of breast cancer, heart failure, or diabetes. AKI is classified as either community-acquired or hospital-acquired. Hospital-acquired AKI predominates in high-income countries, affecting elderly patients with comorbidities and access to advanced care. The primary causes are frequently hospital acquired conditions such as medical procedure, adverse drug reactions and infections. Community-acquired AKI is more common in low-income countries, affecting younger patients, mostly caused by sepsis, dehydration, toxins, and pregnancy, with poor healthcare access specifically women (KELLUM; ROMAGNANI; ASHUNTANTANG; RONCO *et al.*, 2021).

Significant variations exist on current reports of AKI in intensive care unit (ICU) and it is uncertain whether these differences are largely due to differences in the source populations or to methodological differences between studies (SAWHNEY; BELL; BLACK; CHRISTIANSEN *et al.*, 2022). Recently, a multinational cross-sectional study reported AKI in >50% of ICU patients using complete KDIGO criteria (HOSTE; BAGSHAW; BELLOMO; CELY *et al.*, 2015). Another multinational prospective study including children and young adults admitted to ICU reported 26.9% cases of AKI and

11.6% of severe cases of AKI with increase chances of mechanical ventilation, RRT, and death (KADDOURAH; BASU; BAGSHAW; GOLDSTEIN, 2016).

A prospective cohort study in Beijing China evaluated 30 ICUs patients for AKI screening, where they reported 50% cases of AKI with increase rate of mortality, kidney function loss and adverse effects on health in general (JIANG; ZHU; LUO; WEN *et al.*, 2019). AKI is also common among hospitalized patients, it is reported that 10-20% hospitalized patients have AKI (AL-JAGHBEER; DEALMEIDA; BILDERBACK; AMBROSINO *et al.*, 2018; WANG; MUNTNER; CHERTOW; WARNOCK, 2012). Similarly, AKI is found a prevalent issue in pediatric population affecting 27% of critically ill patients and 5% of hospitalized patients (KADDOURAH; BASU; BAGSHAW; GOLDSTEIN, 2017).

The major causes of AKI are divided into prerenal, intrinsic, and postrenal. Prerenal AKI is characterized by reduced glomerular filtration rate (GFR) as result of hypovolemia (due to decreased arterial pressure, hemorrhage, gastrointestinal losses, burns, cirrhosis and capillary leak), decreased cardiac output (due to shock, heart failure, and cardio-pulmonary diseases), sepsis, electrolyte imbalance, and imbalance of intra-abdominal pressure. It may also be induced as a result prescription medication intake such as angiotensin converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs), and nonsteroidal anti-inflammatory drugs (NSAIDS), which often leads to decreased renal perfusion (MERCADO; SMITH; GUARD, 2019; MOLITORIS, 2022).

Intrarenal or intrinsic AKI occurs due to acute tubular necrosis (ATN) (aggravated with sepsis, ischemia or other toxic insults to kidney), vascular diseases (renal artery stenosis, cholesterol emboli, autoimmune medication), glomerular affecting conditions (systemic lupus erythematosus, endocarditis, nephropathy), infections, and medications (antibiotics, NSAIDS) (JACOB; DANNENHOFFER; RUTTER, 2020).

Postrenal AKI is caused by extrarenal obstructions including lower urinary tract issues (calculi, carcinoma, neurogenic bladder, prostate, urethral problems), upper urinary tract problems (pelvic or intra-abdominal tumors, surgical trauma, hematoma), and upper urinary tract intrinsic causes (edema, debris, nephrolithiasis, malignancy) (MERCADO; SMITH; GUARD, 2019).

1.2. Renal ischemia reperfusion injury

Kidney ischemia reperfusion injury (I/R) is a frequent cause of intrinsic AKI in surgical procedures such as kidney transplant, cardiac surgeries and partial nephrectomy. Diseases and health conditions including hypovolemic shock, hypotension, dehydration, and ATN are the other leading causes of kidney I/R induced AKI (LI; LIU; LIU; MENG, 2022; WANG; ZHU; HE; YIN *et al.*, 2019). I/R consists of two phases, first there is organ blood flow restriction (ischemia) for a short period which is followed by restoration of blood flow (reperfusion). The ischemic events result in cell metabolism imbalance leading to tissue hypoxia while reperfusion injury reinstates oxygenation which further aggravates the vicious cycle causing tissue injury and activation of inflammatory cascade (LI; MA; XU; ZHANG *et al.*, 2021).

The hypoxic environment due to ischemia switch aerobic metabolism to anerobic, which results in depletion of ATP level and accumulation of metabolic waste. The reperfusion phase restores oxygenation and aerobic metabolism, however in parallel it leads to generation of reactive oxygen species (ROS). The ROS directly affect cytoskeletal cellular components of functional cells and depletes antioxidant enzymes leading to tissue injury, cytosolic calcium overload, and necrosis (figure 1) (PEFANIS; IERINO; MURPHY; COWAN, 2019; SAAT, T. C.; VAN DEN AKKER, E. K.; IJZERMANS, J. N.; DOR, F. J. *et al.*, 2016).

The accumulating evidence shows that main source of increase ROS generation in I/R is mitochondria. The enzymes responsible to generate ROS both in matrix and membrane of mitochondria includes tricarboxylic acid (TCA) cycle and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase respectively (ZOROV; JUHASZOVA; SOLLOTT, 2014). Mitochondria plays an essential role in ATP synthesis via oxidative phosphorylation. The catabolism of vital organic compounds (carbohydrates, lipids, proteins) yields various products, which enters into mitochondria matrix through TCA cycle producing NADH and $FADH_2$. These two coenzymes carry their electrons and protons into electron transport chain (ETC), a series of multi subunit complexes (Complex I-IV), mobile carriers (coenzyme Q and cytochrome-C), and redox groups located in the inner mitochondrial membrane (SADRI; TOMAR; YANG; AUDI *et al.*, 2023).

The electrons in ETC follows a chain reaction based on redox potential generating a proton electrochemical gradient due to membrane potential ($\Delta\psi$) and a pH gradient (ΔpH) which further influences ATP synthesis through ATP synthase and proton transport (ZOROVA; POPKOV; PLOTNIKOV; SILACHEV *et al.*, 2018). During I/R the mitochondria is affected drastically both structurally and functionally, the reperfusion phase allows the partial recovery of these defects. However, the increased ischemic damage enhance oxygen generated capacity of complex-I and complex-II of ETC allowing increased generation of ROS during reperfusion phase, causing injury of adjacent complexes, dysfunction of ETC and synergizing ROS generation (figure 1) (GRANGER; KVIETYS, 2015; SOLAINI; HARRIS, 2005).

Additionally, among the various factors playing its role in I/R induced AKI, NADPH oxidase 4 (NOX4) has emerged as a significant contributor. Studies have shown that NOX4 is involved in generation of ROS and promoting inflammation; thereby exacerbating kidney injury in I/R induced AKI (MENG; REN; GAO; YANG *et al.*, 2018; PENG; LI; LI; YU, 2022). Furthermore, NOX4 is also linked with increase apoptosis and oxidative stress contributing highly in pathogenesis of I/R induced AKI (PENG; LI; LI; YU, 2022; YANG; GAO; HU; WANG *et al.*, 2021). Targeting ROS generation, scavenging free radicals, stimulating cell survival, increasing the antioxidant enzyme level, and NOX4 are the main strategies to treat and manage kidney I/R (BEACH; PRAG; PALA; LOGAN *et al.*, 2020; FU; WANG; XU; CHEN *et al.*, 2020; LI; WANG; WANG; ZHANG *et al.*, 2023; LI; ZHU; WAN; LI *et al.*, 2021; SAAT, T. C.; VAN DEN AKKER, E. K.; IJZERMANS, J. N. M.; DOR, F. J. M. F. *et al.*, 2016).

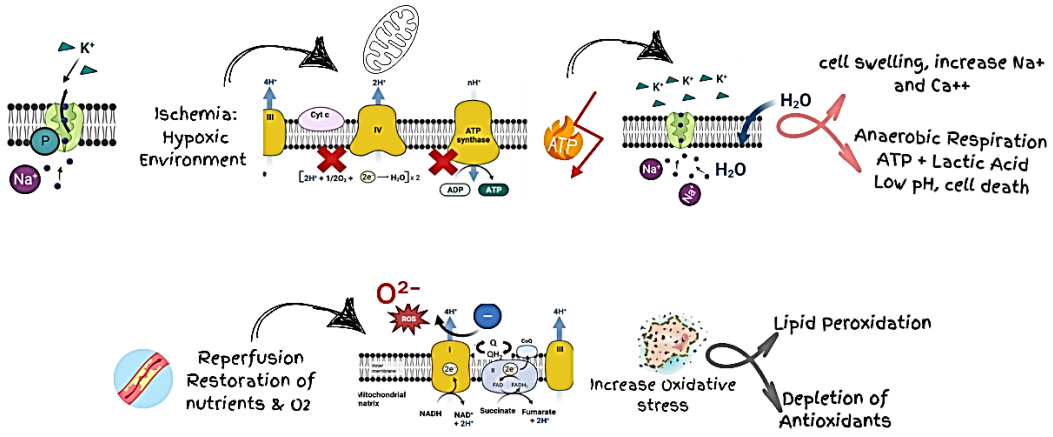

Figure 1: Pathophysiology of kidney ischemia and reperfusion injury: Ischemic conditions lead to ATP depletion, increasing oxidative stress upon reperfusion and aggravating tissue damage.**Source**: Author owns illustration

1.3. Chalcones

Chalcones act as biogenetic precursors for vital classes of phytochemicals including flavonoids and iso-flavonoids (ZAKARYAN; ARABYAN; OO; ZANDI, 2017). Traditionally, chalcones used have been reported in various communities around the globe, e.g., liquorice in China is used for gastro-intestinal, respiratory, and skin ailments. Butein, another chalconoid, is traditionally used for parasitic infections, food additive, painful conditions and gastro-intestinal diseases. In south pacific region, *Boesenbergia rotunda* is used as food spice. In Africa, chalcone rich plant *Lophira alata* is used as for various kind of infections, toothache, contraception and fever (ROZMER; PERJÉSI, 2016).

Structurally, chalcone consist of common scaffold of 1,3-diaryl-2-propen-1-one a three-carbon α, β-unsaturated carbonyl moiety acting as bridge between two aromatic rings. The ring closer to carbonyl group is termed as ring A and other aromatic ring as ring B (figure 2). Chalone occurs in two isomeric forms trans and cis, the trans form of chalcone is thermodynamically more stable comparative to cis form (ZHUANG; ZHANG; SHENG; ZHANG *et al.*, 2017).

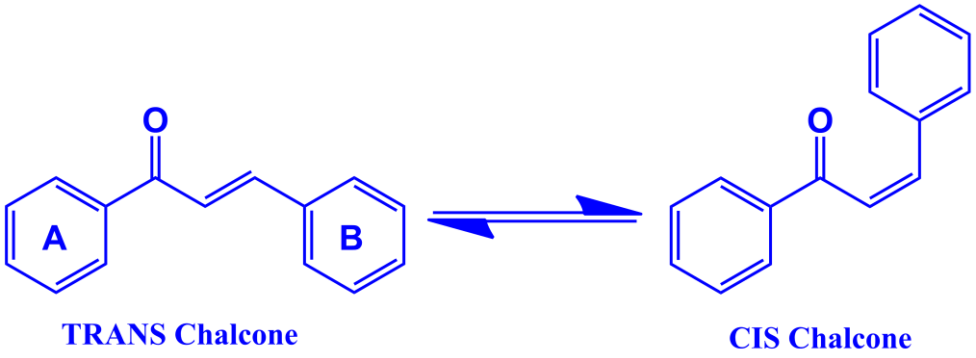

Figure 2: Trans and cis chalcone structures. Trans and cis chalcone structures. The trans form is characterized by a linear arrangement of two aromatic rings linked by a three-carbon unsaturated carbonyl system. It takes on a planar shape with little steric hindrance. The cis form, on the other hand, has a bent structure due to a cis-oriented unsaturated carbonyl system, resulting in enhanced steric interactions and deformed geometry.**Source**: Structure drawn via ChemDraw Ultra 12.0

Chalcone are found abundantly in numerous natural sources including citrus fruits, vegetables, teas, spices, and other plants. Enzyme chalcone synthase in higher plants synthesize chalcones from p-coumarin-CoA and malonyl-CoA. Condensation of 3 molecules of malonyl-CoA results in formation aromatic ring A of chalcone while aromatic ring B and 3C bridge is formed from conversion of amino acid L-phenylalanine to p-coumaril-CoA. After synthesis chalcone contributes in different biochemical pathways of plants nevertheless their main role is acting as precursors virtually for all kind of flavonoids (DÍAZ-TIELAS; GRAÑA; REIGOSA; SÁNCHEZ-MOREIRAS, 2016).

Naturally chalcones are either in aglycones and O-glycosides form, which can modify into various derivatives by variety of substituents such as hydroxyls, methoxy, phenyl and methyl groups (RAMMOHAN; REDDY; SRAVYA; RAO *et al.*, 2020; THAPA; UPADHYAY; SUO; SINGH *et al.*, 2021). In presence of condensing agent, various chalcones derivatives can be synthesized by treating aromatic aldehydes with aryl ketones (ELKANZI, N. A.; HRICHI, H.; ALOLAYAN, R. A.; DERAFA, W. *et al.*, 2022). The chalcone are essential molecules in various fields of science including medicinal chemistry, pharmacology, biochemistry, oncology and many other fields. In last decade more than 100 reviews were published on chalcones covering its key aspects like synthesis, biological activities and synthetic derivatives (MASTACHI-LOZA; RAMÍREZ-CANDELERO; BENÍTEZ-PUEBLA; FUENTES-BENÍTES *et al.*, 2022).

The structure of chalcones is considered as privileged structure as it can be deployed as a primary template to synthesize new derivatives. During the last decade significant amount of research has been conducted on chalcone molecules and its synthetic derivatives. Till date chalcones have demonstrated numerous biological activities including anticancer, antidiabetic, antibacterial, antiulcer, antiplatelet, antiviral, antimalarial, as analgesic, as immunomodulator, anti-inflammatory, antiparasitic, and antioxidant (ELKANZI, N. A. A.; HRICHI, H.; ALOLAYAN, R. A.; DERAFA, W. *et al.*, 2022; ROZMER; PERJÉSI, 2016; SINGH; ANAND; KUMAR, 2014).

ROS plays an important role in I/R pathogenesis and are considered as critical factor in its genesis (GRANGER; KVIETYS, 2015). Therefore, the main interventions to manage I/R induced AKI are targeting ROS generation thus protecting mitochondria, and improving organ function (JIA; WU; PAN; XU *et al.*, 2019; SOARES; LOSADA; JORDANI; ÉVORA *et al.*, 2019).

Since chalcone derivatives have shown potent antioxidant properties and other pharmacological roles, we looked into the possibility of chalcone molecules having protective effects against I/R induced AKI. In this context, 2’-hydroxychalcones were synthesized by Claisen-Schmidt condensation reaction (figure 3). The study targeted important pathogenic processes in I/R, including ROS generation, early apoptosis, necrosis, and the antioxidant system.

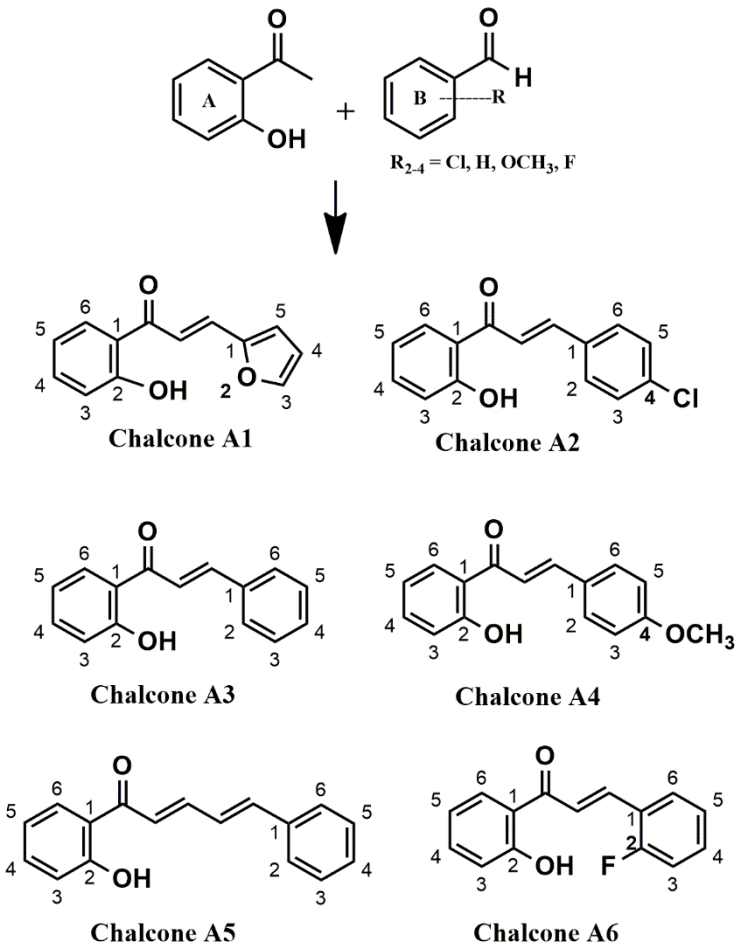

Figure 3: Synthesis of chalcone molecule derivatives via Claisen-Schmidt condensation reaction.

Source: Structure drawn via ChemDraw Ultra 12.0

2. Justification and relevance

There are an accumulative evidences regarding the drastic impact of I/R induced AKI on healthcare system effecting significant amount of populations both in hospitals and community (NIEUWENHUIJS-MOEKE; PISCHKE; BERGER; SANDERS *et al.*, 2020; SIMMONS; SCHREIBER; GILL, 2008). Current literature data show significant number of protective strategies against I/R induced AKI, specifically studies with plant-based therapies are on the rise. I/R is a key cause of AKI in different clinical settings, including kidney transplantation and diseases like hypovolemic and septic shock (KIERULF-LASSEN; NIEUWENHUIJS-MOEKE; KROGSTRUP; OLTEAN *et al.*, 2015).

Oxidative stress plays a critical role in the development of I/R induce AKI. The kidney, rich in angiogenesis and metabolic activity, is predominantly vulnerable to the negative effects of ROS (YU; LIU; WANG; ZHANG *et al.*, 2021). In all types of AKI, oxidative stress plays a major role in organ failure, highlighting its significance in the pathophysiology of kidney injury (DUBE; MATAM; YEN; MANG *et al.*, 2017). Cell apoptosis is one of the injury effects on kidney tubules and endothelial cells, which restrict blood flow and cause inflammation and fibrosis (HATCHER; TESFAY; TORTI; TORTI, 2015). Antioxidants like N-acetylcysteine (JIANG; GE; IM; ENGLAND *et al.*, 2018) and CDDO-imidazolide (LIU; REDDY; HIGBEE; POTTETI *et al.*, 2014) have shown promising results in protecting I/R induced AKI. Similarly, antioxidants like erythropoietin (SONG; LEE; YOU; CHIN *et al.*, 2009) and visomitin (SONG; SHENG; LEI; GAN *et al.*, 2022) are also being studied in clinical trials for their ability to prevent and treat AKI, demonstrating the continuous attempts to create efficient treatments. Moreover, during I/R induced AKI there is upregulation of NOX4, which further augment the oxidative stress environment. The increase expression of NOX4 is associated with apoptosis and fibrosis (PENG; LI; LI; YU, 2022). Furthermore, NOX4 is linked with increase TLR4 cell signaling causing increase generation of ROS and activation of cellular death events (CHEN; NAN; YANG; XIAO *et al.*, 2023).

However, treating I/R induced AKI is still a challenge as there is lack of definite treatments, the intervention is mainly based on management and symptomatic treatment (CHATAURET; BADET; BARROU; HAUET, 2014). Moreover, chalcones with strong antioxidant action along with other pharmacological actions have not been properly

explored for treatment of I/R induced AKI. Therefore, there is immense need to explore new therapies in order to provide protection against drastic effects of I/R induced AKI.

The methodology we applied in this study are already established and well recognized (SAMPAIO; DA COSTA; MENESES; ARRIETA *et al.*, 2016). Moreover, use of HK-2 cells provides controlled and reproducible environment to evaluate the protective role of chalcone molecules. The main pathophysiological pathways that are involved in I/R injury were targeted in HK-2 cells, minimizing the use of animal's models while still providing valuable insights into cellular responses.

3. Objectives

3.1. General objective

Investigating the potential protective effect of 2'-hydroxychalcones against ischemic reperfusion induced acute kidney injury in *in-vitro* model of HK-2 cells.

3.2. Specific objectives

- To analyze the optimum and protective concentration of 2'-hydroxychalcones (A1-A6) on HK-2 cells.
- To analyze the protective effect of 2'-hydroxychalcones against ischemia reperfusion injury.
- Study effect of chalcone derivative on mitochondria transmembrane potential and generation of reactive oxygen species against I/R AKI.
- Study effect of chalcone derivative on antioxidant enzymes level.
- To analyze effect of I/R and chalcone derivative on cytological features of HK-2 cells via scanning electron microscope.
- Study the potential interaction of chalcone derivative with NOX-4 via molecular docking.

4. Material and Methods

4.1. Synthesis and chemical characterization of chalcones

Chalcones derivatives (A1-A6) were kindly donated by professor Hélcio Silva dos Santos of State University of Vale do Acaraú, which were synthesized from 2-hydroxyacetophenone via Claisen-Schmidt condensation reaction. The complete procedure of synthesis is described in detail in Xaveir *et al.* 2021 including its key steps and reaction conditions to obtain the desired chalcone derivatives (DA CUNHA XAVIER; DE ALMEIDA-NETO; ROCHA; FREITAS *et al.*, 2021).

Briefly, equimolar quantities of 2-hydroxyacetophenone and benzaldehyde or its derivatives were dissolved in ethanol followed by addition of sodium hydroxide (NaOH) solution, after 48 hours it resulted in precipitation of chalcones (A1-A6) at ambient temperature (figure 3). The chalcones were purified by filtering, washing with cold water, drying, and recrystallizing from ethanol. A solitary spot on thin-layer chromatography (TLC) validated the purity. The chalcones derivatives were then submitted for characterization evaluated via nuclear magnetic resonance (NMR), FT-Raman, ATR-FTIR, and UV-Vis (DA CUNHA XAVIER; DE ALMEIDA-NETO; ROCHA; FREITAS *et al.*, 2021).

4.2. Cell culture assay

HK-2 cells were sourced from the Rio de Janeiro Cell Bank (BCRJ). The cells were then grown using Dulbecco's Modified Eagle's Medium (DMEM) (manufactures by Invitrogen, USA) in plastic containers. The DMEM was enhanced with a number of necessary elements including glutamine, sodium bicarbonate (3.7 g/L), glucose, sodium pyruvate, inorganic salts, vitamins, and amino acids. To further guard against bacterial contamination, the culture medium was supplemented with streptomycin (130 mg/ml) and penicillin (200 IU/ml).

The cells were maintained at 37 °C in 5% CO_2 humidified incubator until they reached full confluence. The cells were then detached with 0.25% trypsin-EDTA solution, cells were then centrifuged, supernatant was discarded, and pellet was resuspended in 1 ml of DMEM media. The cells were counted via Neubauer counting chamber and then seeded at concentration of 1×10^5 cells/ml in 96 or 24 wells plates overnight to allow

adherence and proliferation (figure 4) (RYAN; JOHNSON; KIRK; FUERSTENBERG *et al.*, 1994). For each experiment the same process was repeated the cells were maintained in 10 % DMEM media at 37 °C in 5% CO_2 humidified incubator. If any signs of cells contamination were found at any stage of experiment, the cells were discarded and new stock of cells were then utilized.

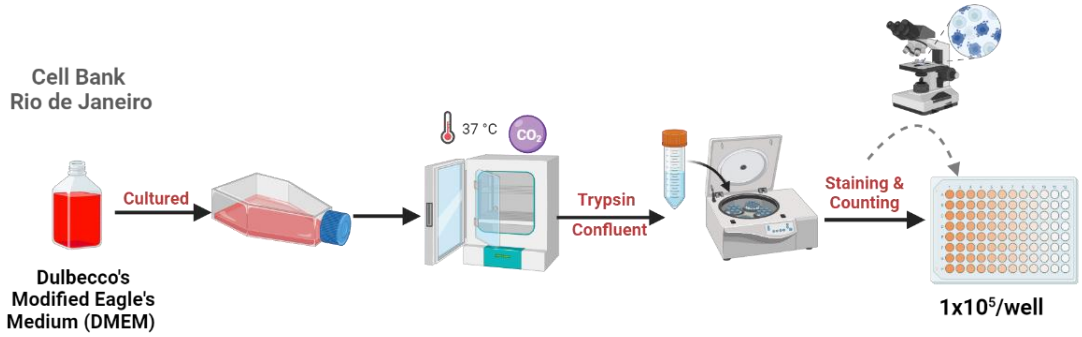

Figure 4: Thematic presentation of HK-2 cells culture.

Source: Author owns illustration

4.3. *In-vitro* ischemia reoxygenation model

For inducing *in-vitro* ischemia reoxygenation, the anaerobic chamber method was used as mentioned in (SAMPAIO; MENEZES; LIMA; COSTA SILVA *et al.*, 2019). This model mimics the same mechanism of natural ischemia reperfusion phenomenon involving hypoxia and reoxygenation. The ischemia was induced by replacing the DMEM with glucose, pyruvate and FBS deficient DMEM followed by placing the plates in anaerobic chamber for 24 hours. Reoxygenation was then induced replacing deficient DMEM with complete DMEM and incubated for 3 hours in oxygen free incubator followed by chalcone molecules treatment at various concentrations (250; 125; 62.5, 31.25 and 15.62 µM) for 24 hours (figure 5).

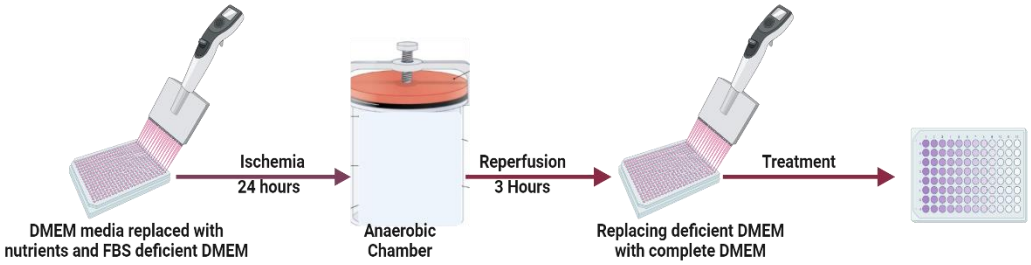

Figure 5: Representation of an ischemia-reoxygenation induced acute kidney injury *in-vitro* model for studying effect of chalcones.

Source: Author owns illustration

4.4. Cytotoxicity assay

It is a colorimetric assay for determination of viable cells ability to transform soluble tetrazolium salt 3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into insoluble purple color formazan crystals. The tetrazolium salt MTT has the unique ability to readily accept electrons from oxidized substrates including NADH and NADPH. This chemical transformation of MTT leads to conversion of its initial yellow color into purple color formazan crystals, which can be solubilized with an organic solvent. The concentration of this solvent can be quantified via spectrophotometry in order to precisely measure the reaction extent (SUPINO, 1995).

To evaluate optimum concentration of chalcone derivatives for the said study, HK-2 cells were treated with chalcone derivatives **(A1-A6)** with different concentrations (250; 125; 62.5, 31.25 and 15.62 µM) for 24 hours. The cells viability was assessed by treating with MTT solution at concentration of 2.5mg/ml following MTT assay standard protocol (MOSMANN, 1983). The cells were then incubated for 3 hours at 37 °C in 5% CO_2 humidified incubator resulting in formazan crystals inside cells followed by treatment with 10 % SDS solution to dissolve the formazan crystals.

After 17 hours, the cells absorbance was read at 570 nm (figure 6). Cell viability was calculated in comparison to negative control groups that were treated with PBS only via non-linear regression method. Among the chalcone derivatives (A1-A6) the one which show pronounced effect on HK-2 cells while maintaining non-toxic concentration was selected for further experiments.

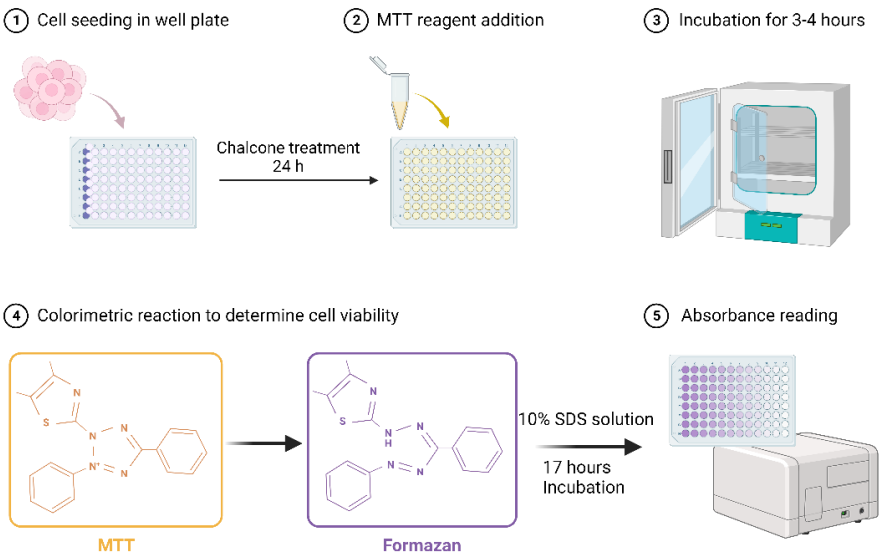

Figure 6: MTT assay methodology. (1) Cells were seeded in 96 well plate in concentration 1x10^5 cells per well (2) After Ischemia reoxygenation exposure, cells were treated with MTT reagent and (3) incubated for 3 hours. (4) Allowing MTT transformation into formazan crystals in this incubation period. (5) Formazan was solubilized using 10% SDS solution and plate absorbance was measured via spectrophotometer at 570 nm frequency. **Source**: Author owns illustration

4.5. Flow cytometry assays

Flow cytometry is a technique that uses lasers to evaluate single cells in suspension, recording scattered and fluorescent light signals that are subsequently converted into electronic data, allowing for the investigation and purification of cell populations depending on their optical characteristics. The efficiency of this technique depends on the light-scattering characteristics that the cells under investigation display. The obtained information can offer important new perspectives on the molecular, biological, and physical properties of the particles. The structural and morphological characteristics of the cell are reflected in light scattering, and the amount of bound fluorescent probes on the cell or its constituent parts is shown by fluorescence emission (ADAN; ALIZADA; KIRAZ; BARAN *et al.*, 2017).

We conducted cellular analysis using flow cytometer to assess three key parameters: apoptosis and necrosis, cytoplasmic reactive oxygen species generation, and mitochondrial transmembrane potential. This evaluation aimed to understand the impact of both I/R and the chalcone derivative on HK-2 cells.

For each parameter's analysis, HK-2 cells were cultured in 24 wells plate at concentration of 1x10^5/ ml per well. For normal control group separate plate was used

while cells exposed to I/R separate plate was used. After 24 hours, I/R exposed plate was then treated with chalcone A4 at two different concentrations (31.25 and 15.62 μM) based on MTT analysis, for 24 hours. Then trypsin was added to detach the cells from wells, followed by 3 times centrifugation (4000 RPM, 5 minutes) and washing with phosphate buffer. The fluorescence intensity was measured using flow cytometer FACSCalibur (BD Biosciences, New Jersey, USA), using Cell Quest Pro ™ software.

4.5.1. Necrotic and apoptotic cells analysis

Cell viability and cell death progression is essential analysis in various biological and medical research fields. The use of Annexin V-Phycoerythrin (AxPE) along with propidium iodide (PI) is recognized as gold standard approach for assessing cell death, which have enabled the researchers to distinguish between cells in the dying phase, viable state, and dead state. The AxPE differentiates between viable and apoptotic cells. The mechanism involved is based on phosphatidylserine (PS) exposure on cells surface of cells. The PS is expressed on cells undergoing apoptosis as a result of caspase activation, which allows the binding of AxPE. Contrary viable cells typically express very low level of PS therefore the extent of annexin-V staining serves as differentiation between apoptotic and viable cells (JIANG; TIXEIRA; CARUSO; ATKIN-SMITH *et al.*, 2016).

While PI dye is used to differentiate apoptotic cells that has permeabilized membranes (late apoptotic or secondary necrotic cells). So, via this approach we can identify apoptotic and secondary necrotic cells from viable cells (KOÇ; ÇELIK-UZUNER; UZUNER; ÇAKMAK, 2018; VAN GENDEREN; KENIS; LUX; UNGETH *et al.*, 2006).

To identify the extent of viable, non-viable and secondary necrotic cells, primarily the cells were treated with chalcone derivative for 24 hours and then detached with 0.25% trypsin EDTA solution followed by centrifugation, two times washing with binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.2) and then labeled with PI and AxPE according to manufacturer instructions (ARANDA; SEQUEDO; TOLOSA; QUINTAS *et al.*, 2013). The experimental groups were then analyzed via flow cytometer at the rate of 10,000 events per analysis. The percentage of unlabeled, PI labeled, and AxPE labeled and doubled labeled both with PI and AxPE were analyzed, recorded and

compared with each other. The mechanism of these dyes staining has been depicted in figure 7 below.

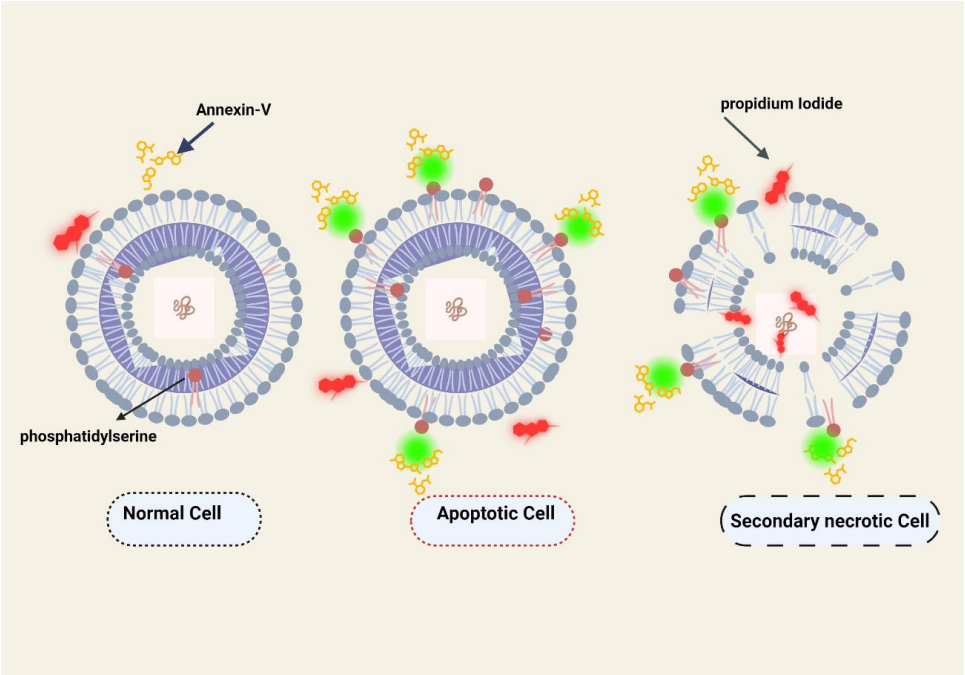

Figure 7: The fluorescence mechanism of AxPE and Propidium Iodide. AxPE labeled cells show green fluorescence, suggesting early apoptosis, whereas Propidium iodide-stained cells show red fluorescence, signifying apoptosis or necrosis. Separating necrotic and apoptotic cell populations can be accomplished by co-staining using Propidium Iodide and AxPE.

Source: Author owns illustration

4.5.2. Cytoplasmic reactive oxygen species (ROS) generation analysis

There are numerous methods to measure ROS generation. The most basic approaches include cell-permeable fluorescent and chemiluminescent probes, notably, 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA) is widely employed for directly measuring cell redox states. Its use has several merits including ease of use, high sensitivity to redox state changes, low cost, and ability to track ROS fluctuations over time (ERUSLANOV; KUSMARTSEV, 2010).

The principle behind it is its lipophilic nature, enabling DCFH-DA to readily pass through the plasma membrane, where it becomes a substrate for cytoplasmic esterase's. The intracellular esterase's cleave the ester bonds of DCFH-DA, resulting in the polar, non-fluorescent H_2DCF that accumulates within cells. Following oxidation, the extremely fluorescent DCF is produced (figure 8).

The redox status of the sample is determined by monitoring the fluorescence increase. Using a flow cytometer, the fluorescence increase at 530 nm with illumination at 485 nm is quantified. The green fluorescence emitted by DCFH-DA is proportional to the concentration of ROS present inside the cells (ARANDA; SEQUEDO; TOLOSA; QUINTAS *et al.*, 2013; ERUSLANOV; KUSMARTSEV, 2010).

To measure the generation of cytoplasmic ROS, 2',7'-dichlorofluorescin diacetate DCFH-DA dye was added at 100 μM concentration to HK-2 cells after 3 hours of treatment. The cells were then incubated, detached with 0.25% trypsin EDTA solution, washed twice and then analyzed via flow cytometer. The results of analysis were recorded as relative fluorescence intensity, which was determined by dividing fluorescence intensity mean values of experimental groups by fluorescence intensity mean values of control groups.

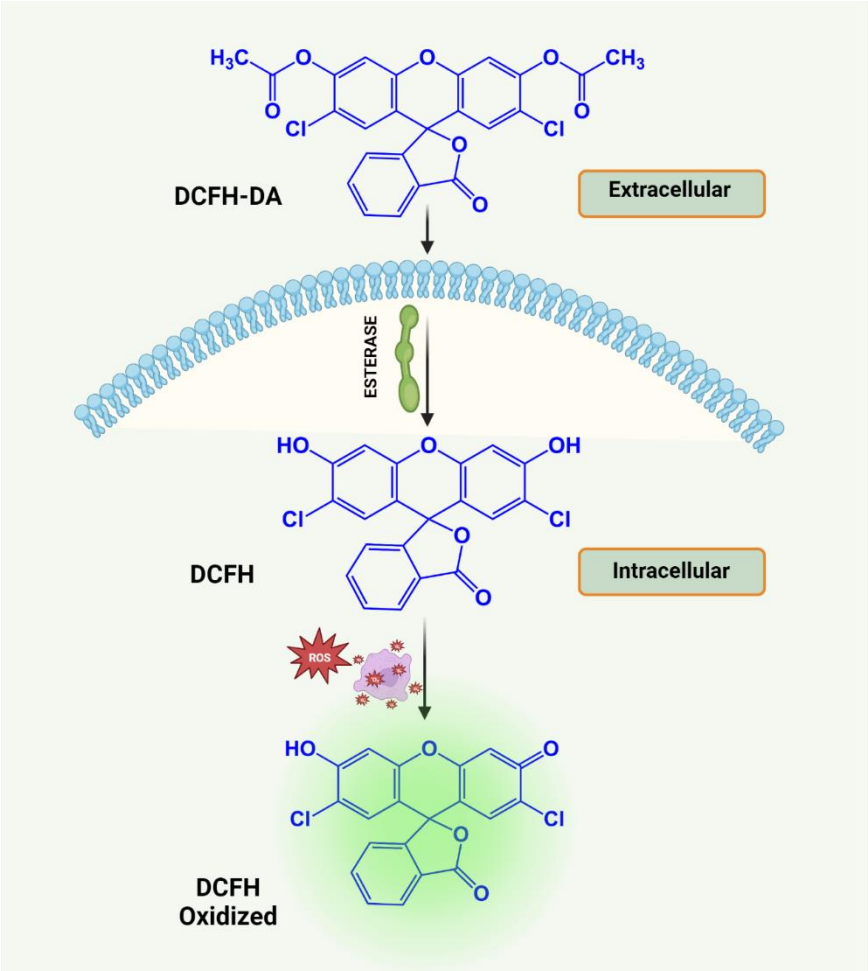

Figure 8: The DCFH-DA fluorescence principle. DCFH-DA upon entering the cell, cellular esterase's convert it into DCFH. In the presence of cytoplasmic reactive oxygen species (ROS), DCFH undergoes oxidation, producing the fluorescent compound DCF. Fluorescence intensity increases as a result of cellular oxidative damage.**Source**: Author owns illustration

4.5.3. Mitochondrial transmembrane potential (ΔΨm) analysis

Mitochondrial membrane potential (ΔΨm), generated during oxidative phosphorylation, is essential for ATP production. Maintaining stable ΔΨm and ATP levels is critical for cell health. Prolonged deviations can harm cell viability and lead to various pathological challenges, including the selective removal of dysfunctional mitochondria. ΔΨm also facilitates the transport of essential ions and proteins for mitochondrial health (ZOROVA; POPKOV; PLOTNIKOV; SILACHEV *et al.*, 2018).

Numerous cationic dyes including rhodamine 123 (Rho123), which was the first widely utilized for this purpose and continues to be prevalent in research. Rho123 can accumulate in mitochondrial matrix due to its lipophilicity and its cationic nature which

respond to electric potential across the inner mitochondrial membrane. Upon entering into mitochondria rho123 acts as substrate for mitochondrial esterase's converting it into cation zwitterion (figure 9) (ZOROVA; POPKOV; PLOTNIKOV; SILACHEV *et al.*, 2018).

When mitochondria become energized, the Rho123 fluorescence spectrum shifts to the red, and there is an empirical linear relationship between the change in fluorescence intensity and the membrane potential. This linear relationship is then used to quantify membrane potential in mitochondria, as established previously (HUANG; CAMARA; STOWE; QI *et al.*, 2007; O'CONNOR; VARGAS; KIMLER; HERNANDEZ-YAGO *et al.*, 1988).

In our experiment mitochondrial depolarization was evaluated through alteration of the ΔΨm using rhodamine 123 (Rho123) dye. The cells were then treated with chalcone derivative followed by PBS wash and staining with Rho123 (10 µg/ml) dye for 30 minutes. Before submitted to flow cytometer, cells were washed two times with PBS. The ΔΨm was then measured from the fold changes of fluorescent intensity. The results were calculated from the mean values of fluorescent intensity of experimental groups and control groups.

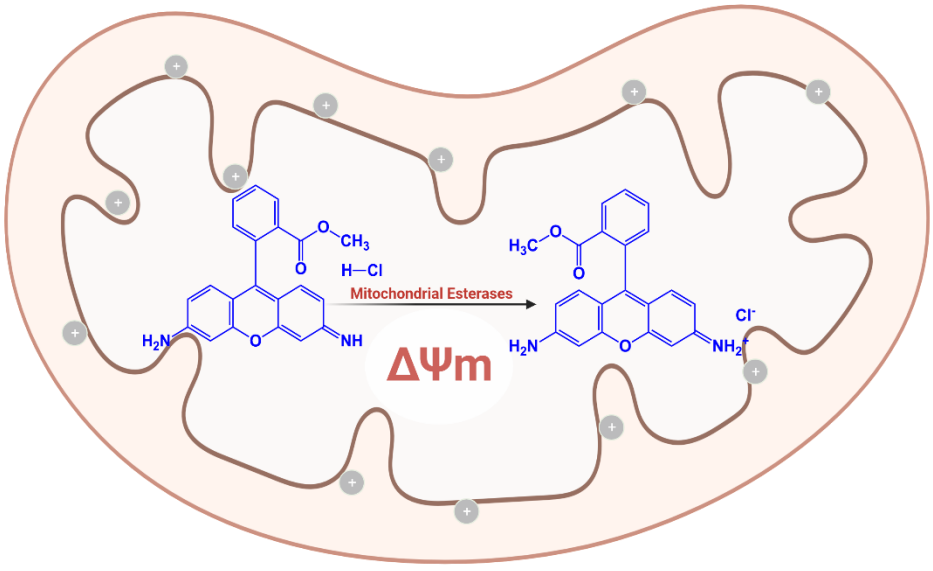

Figure 9: Illustrating Rhodamine123 mitochondrial transmembrane potential detection mechanism.

Source: Author owns illustration

4.6. Oxidative stress markers analysis

For this purpose, cell lysate was extracted. Briefly, cells were cultured followed by treatment, washing with ice cold PBS, suspending in buffer (5 mM EDTA, 0.1% Triton X-100 and 0.6% sulfosalicylic acid (pH 7.5), then frozen in freezer in order to allow complete rupture of cell membrane. The suspension was then centrifuged at 3000g for 4 minutes, the supernatant was separated, and protein content was measured according to Bradford method (BRADFORD, 1976). After that supernatant (cell lysate) was stored at -80 °C for analyzing thiobarbituric acid reactive substances (TBARS), glutathione (GSH) and superoxide dismutase (SOD) as explained below.

4.6.1. Thiobarbituric acid reactive substances (TBARS) analysis

Among the various biological consequences of oxidative stress, lipids emerge as the primary category of biomolecules affected. The principal and most studied consequence of polyunsaturated fatty acid peroxidation is malondialdehyde (MDA). This aldehyde possesses high toxicity and warrants recognition beyond being solely an indicator of lipid peroxidation. Potential mutagenic and atherogenic effects have been linked to its interactions with proteins and DNA (DEL RIO; STEWART; PELLEGRINI, 2005).

MDA is measured as part of the thiobarbituric acid-reactive substances (TBARS) for monitoring lipid peroxidation. MDA interact with TBA under optimum conditions, resulting in a red-colored, light-absorbing, and fluorescent TBA-MDA condensation adduct abbreviated as TBA-MDA or MDA-(TBA)2 (figure 10) (TSIKAS, 2017; VALENZUELA, 1991).

For this purpose, 100 µL of cell lysate was mixed with 40% trichloroacetic acid and 400µL of 60% thiobarbituric acid according to manufacturer instructions. The mixture obtained was then incubated at 95 °C for 40 minutes followed by placing in ice bath. To stop the reaction glacial acetic was added to the mixture. The mixture was then centrifuged and read at 530 nm in spectrophotometer. TBARS concentration was measured from standard malondialdehyde (MDA) and cell proteins curves (MIHARA; UCHIYAMA; FUKUZAWA, 1980).

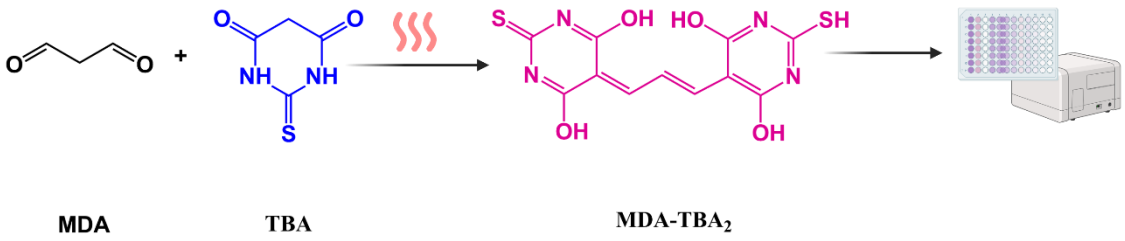

Figure 10: Illustrating thiobarbituric acid reactive substances (TBARS) formation. In the reaction, TBARs and malondialdehyde (MDA) combine to generate a measurable colored complex that serves as a gauge for lipid peroxidation.**Source**: Adopted from (DE LEON; BORGES, 2020)

4.6.2. Glutathione (GSH) evaluation

Reduced glutathione (GSH) is a vital intracellular thiol, acting as a non-enzymatic antioxidant against ROS and nitrogen species generated during various cellular processes. Along oxidized glutathione disulfide (GSSG) it prevents essential biomolecules from oxidative damages. In these biochemical reactions, GSH is oxidized to produce GSSG, which is then converted back to GSH with the help of NADPH-dependent glutathione reductase (figure 11). GSH also serves as a cofactor for detoxifying enzymes, such as glutathione peroxidases (WU; FANG; YANG; LUPTON *et al.*, 2004).

In our experiment, reduced glutathione (GSH) level was measured by taking 200 µL of cell lysate and mixed with 400 µL of Tris-HCL buffer and 10 µL 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). Then mixture absorbance was measured at 412 nm frequency in spectrophotometer. The final calculated results were expressed as nmol GSH/mg protein (SHARMA; NEMECZ; ZHU; STEELE, 1997).

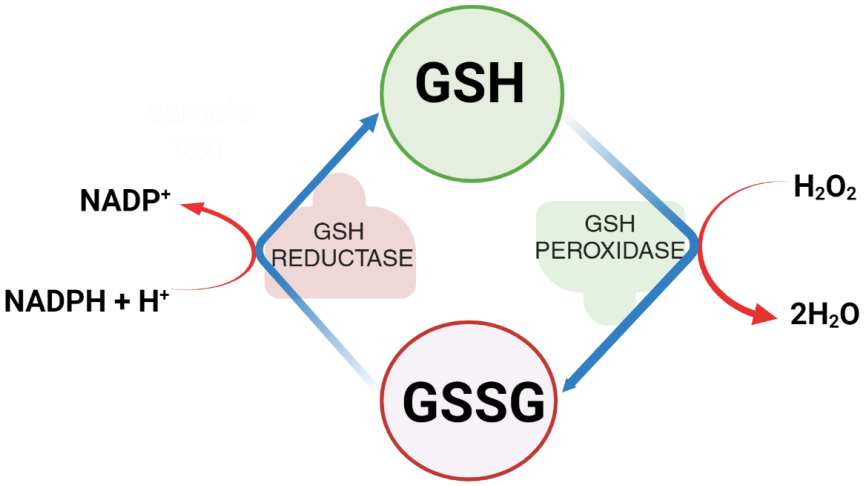

Figure 11: Representation of the enzymatic conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH), facilitated by NADPH.This process involves the reduction of GSSG to GSH, accompanied by the conversion of NADPH to NADP+ and the detoxification of hydrogen peroxide (H2O2) to water (H2O), demonstrating the interrelated roles of both molecules in cellular redox homeostasis.**Source**: Adopted from (POLADIAN; NAVASARDYAN; NARINYAN; ORUJYAN *et al.*, 2023)

4.6.3. Superoxide dismutase (SOD) accumulation analysis

Superoxide dismutase (SOD) plays a catalytic role in dismutation of oxygen radicals O_2^- into H_2O_2 and O_2. The efficient structure of superoxide dismutase allows it to interact with O_2^- at rates that are close to diffusion limitations. A transition metal ion at the active site facilitates its catalytic activity. This function is performed by three metals: manganese, iron, and copper. Zinc is another element found in copper-containing enzymes. It helps with the effective dissociation of the H_2O_2 product generated during O_2^- dismutation, although it is not directly engaged in catalysis. A decrease in SOD activity can have far-reaching effects on cellular functions, including deficiencies in amino acid metabolism and increased levels of oxidative DNA damage. (CULOTTA, 2001).

For Superoxide dismutase (SOD) measurement, 30 µL of cell homogenate was mixed with 99 µL PBS, 6µL of MTT at concentration 1.25 mmol/L, and 15µl of pyrogallol at concentration of 0.1 mmol/L, in 96-well microplate. The plate was then incubated at 37 °C for 10 minutes followed by addition of 150 µL dimethyl sulfoxide to stop the

reaction. Then absorbance was read at 570 nm frequency in spectrophotometer. The relative accumulation of O2- was expressed as nmol/mg protein.

4.7.Scanning electron microscopy

The methodology involves the disposition of a specialized microscope equipped with both electrons and ion columns. The column of electrons was adeptly angled towards a comparable column of ions, the SEM creates a controlled stream of positively charged ions directed towards the specimen. This dynamic interaction between the electron and ion columns enables scanning of the sample's surface, resulting in intricately detailed and extremely accurate three-dimensional pictures (figure 12) (SMITH; STARBORG, 2019).

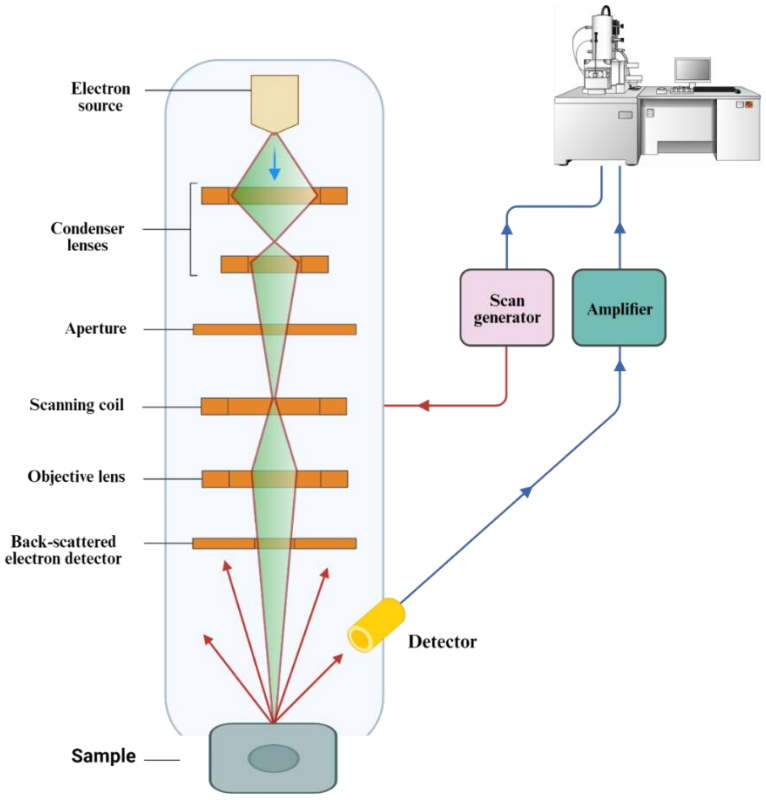

Figure 12: The imaging process of a scanning electron microscope (SEM) is demonstrated, starting with the sample's pretreatment with a conductive coating and ending with the electron beam moving across the sample's surface to create a topographical image that is detailed. **Source:** Author owns illustration

For the experimental arrangement, HK-2 cells were plated at a concentration of $1x10^5$ cells/ml in a 24-well plate. Each well contained a circular glass lamina at the bottom to facilitate cell adhesion. Following a 24-hour interval, chalcone A4 was added to the

wells at concentrations of 31.25 and 15.62 μM, based on their satisfactory results in the MTT assays.

Subsequently, the cells were fixed for 2 hours with 2.5% glutaraldehyde in PBS (Electron Microscopy Sciences, Hatfield, Pennsylvania). They were then washed twice with PBS, twice with distilled water, and subjected to centrifugation (800 G/10 min). In order to prepare the samples for SEM, a sequence of ethanol dehydration steps was applied, gradually increasing the ethanol concentrations (30, 50, 70, 90, and 100%). The samples were left to dry inside a desiccator containing silica gel overnight. To enhance conductivity, the samples were then coated with a layer of gold and examined using a Quanta 450 FEG-FEI scanning electron microscope (Oregon, USA) (MELLO; BOOK; NICOLAS; OTTO *et al.*, 2017). The resulting digital electro-micrographs were stored in a computer at the Analytical Center - UFC, utilizing the Nis 4.0 Software.

4.8. Molecular docking assay

The geometrical optimization of the selected chalcone molecule (4A) was carried out within the scope of the Density Functional Theory (DFT) with B3LYP/6-311++G (d,p) computational level (DITCHFIELD; HEHRE; POPLE, 1971) in the gas phase using the Gaussian 09 software (FRISCH; TRUCKS; SCHLEGEL; SCUSERIA *et al.*, 2009) and the Avogadro 1.2.0 software (HANWELL; CURTIS; LONIE; VANDERMEERSCH *et al.*, 2012) to draw the input molecules.

Molecular docking simulations were performed to evaluate the theoretical interaction of chalcone 4A with NADPH oxidase compared to the site occupied by apocynin, an efficient inhibitor of NADPH oxidase (KANEGAE; CONDINO-NETO; PEDROZA; DE ALMEIDA *et al.*, 2010). In addition, the three-dimensional structure of p47phox auto inhibited from NADPH oxidase was obtained from the Protein Data Bank repository (PDB 1NG2).

The identification of a potential binding site between the optimized chalcone molecule and NADPH oxidase was performed by molecular docking with the Auto docking Vina software (TROTT; OLSON, 2010), where the generated grid parameters were centralized to involve the entire protein structure using the axes (25,765x, 42,613y, 11,711z), size (92x, 108y, 120z) with NOX4 (p47phox). Molecular graphs were

constructed using the UCSF Chimera 1.8 software package (PETTERSEN; GODDARD; HUANG; COUCH *et al.*, 2004).

4.9. Statistical analysis

All the experiments were performed in triplicates. The results were expressed as means ± standard error of mean (S.E.M). The statistical analysis was performed in GraphPad Prism version 8.4.3 (USA) software, primarily the data was normalized followed by one way ANOVA and Bonferroni test for comparison between groups.

5. Results

5.1. Synthesis and characterization of chalcones

Six hydroxychalcones (A1-A6) were synthesized from 2-hydroxyacetophenone and benzaldehyde or its derivatives via Claisen-Schmidt condensation reaction as shown in figure 3. The main substitutions were on its second ring, chalcone A1 contain furan ring, A2 contain a chlorine atom on position 4, A3 was without any substitution, A4 contain a methoxy group on position 4, A5 consist of an extra double bond in its chain and A6 has fluorine substituent on position 2 **(**figure 3).

The chalcones derivatives have already been characterized in study executed by "da Cunha Xavier et al., 2021", therefore here we will briefly describe it main features. The NMR spectrum of chalcone derivatives showed confirmatory signals of E-stereochemistry corresponding to α and β hydrogens of the double bond. The spectrum also has matching signals corroborating the aromatic hydrogens in first ring and second ring along with olefinic protons signals. The chalcone A4 NMR study confirmed the presence of methoxy group and trans-arrangement of olefinic protons (DA CUNHA XAVIER; DE ALMEIDA-NETO; ROCHA; FREITAS *et al.*, 2021).

The vibrational study of chalcone molecules A1-A6 reveals their unique properties due to their diverse atomic compositions and nonlinear architectures. Changes in the B-ring substitution pattern, including adjustments at the 4-position and enone chain elongation, cause spectral shifts in the Raman and infrared bands. Because of the chalcones' limited symmetry inside the C1 point group, the experimental Raman and infrared spectra show a significant correlation. Unique stretching and bending modes, such as -C=O, -C=C-, and -C-H stretching, are seen, as well as unique bands verifying particular ring topologies. Hydroxyl and carbonyl stretching modes display patterns unique to individual chalcones. In chalcones A2 and A6, electronegativity has an effect on vibrational modes.

Using B3LYP/6-311G (d,p) theory, the HOMO and LUMO border molecular orbitals of chalcones A1-A6 display different electrical properties. Except in A6, where it extends to the A-ring, the HOMO is mostly restricted to B-rings and enone chains. Differences in electron density influence hydrogen bond strength, implying stronger hydrogen bonds in A6. Except for the methyl group in A4, the LUMO is located over aromatic carbon and oxygen atoms and is localized over chloride and fluorine atoms in

A2 and A6. These orbitals reveal reactivity information such as the energy gap, ionization energy, electron affinity, and other chemical characteristics. Chalcone A5 has a high reactivity and an electrophilic property. Because of the presence of chlorine and fluorine, electronegativity and ionization energy are increased in A2 and A6 (DA CUNHA XAVIER; DE ALMEIDA-NETO; ROCHA; FREITAS *et al.*, 2021).

5.2. Cytotoxicity assay

Several concentrations of chalcone derivatives (A1-A6) were assessed thorough MTT assay. The HK-2 cells were treated with different concentrations of each chalcone molecule (250, 125, 62.5, 31.25 and 15.62 μM). The chalcone molecules showed concentration dependent cell cytotoxicity (figure 13A).

HK-2 cells that were exposed to ischemia and reoxygenation (I/R), treatment with chalcone A4 at concentrations (31.25 and 15.62 μM) have significantly increased the cell viability of those cells compared to I/R control group showing its protective action. While, other chalcone derivatives (A1, A2, A3 and A5) did not provide any protection against ischemic reperfusion injury (figure 13B). The chalcone A6 was able to provide protection at only one concentration 15.62 μM.

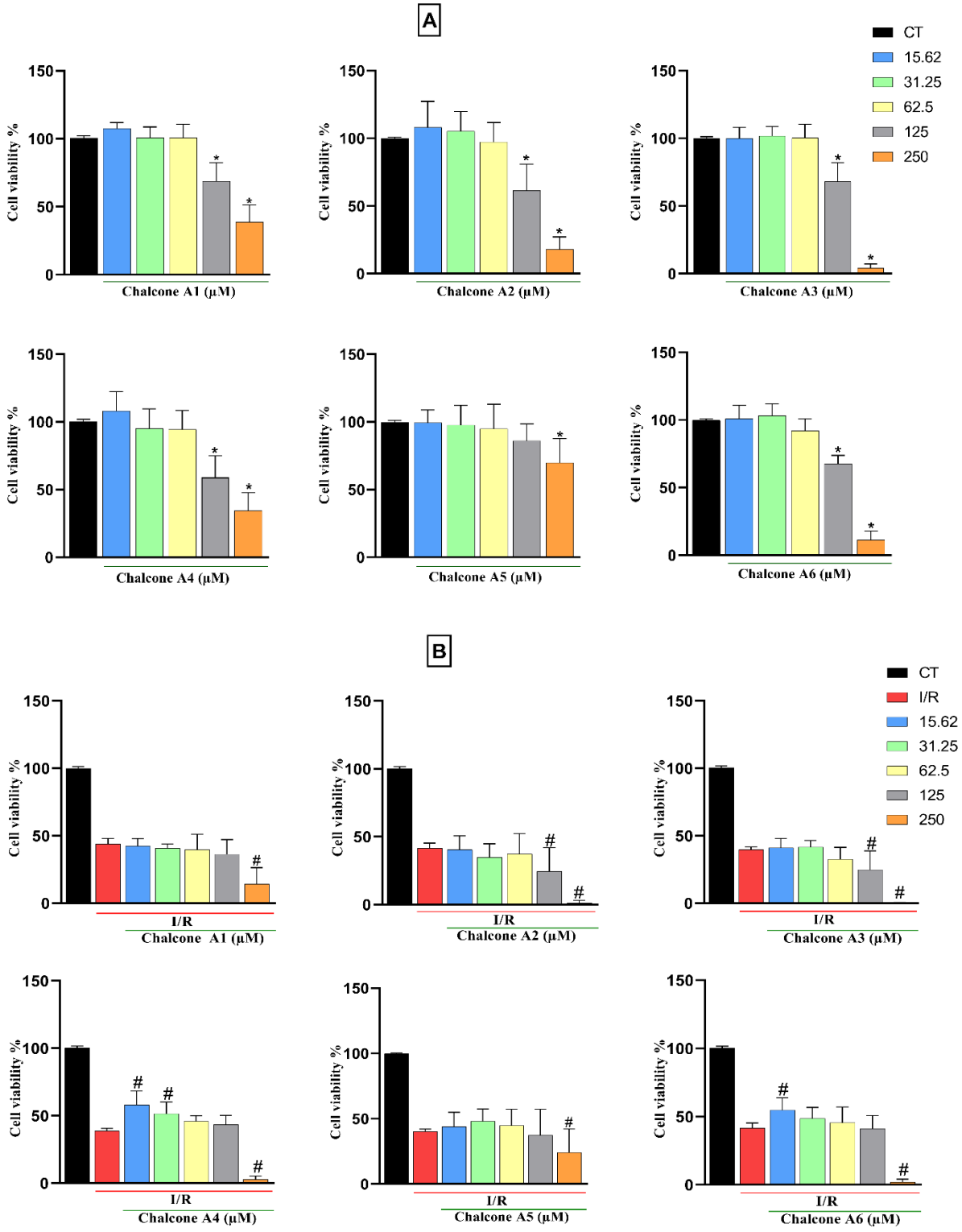

Figure 13: Toxicity range of Chalcone molecule (A1-A6) at different concentration on HK-2 cells. (A) MTT assay of HK-2 cells treated with chalcones at aerobic condition (B) MTT assay of HK-2 cells treated with chalcones and exposed to ischemia reoxygenation.

Source: Research data

5.3.Flow cytometry assays

5.3.1. Analysis of apoptotic, necrotic and non-viable cells

The cell death mechanisms and protective action of chalcone A4 were evaluated via flow cytometry. Different groups of cells were treated with PI, among which I/R control group shows high percentage of PI (24.53%). While the cells treated with two different concentrations (31.25 and 15.62 μM) of chalcone A4 has reduced the percentage of PI fluorescence (Concentration 1: 22.63% and Concentration 2: 16.29%) showing decreased amount of cell death events compared to I/R group receiving no treatment (24.53%). Similarly, HK-2 cells treated with chalcone A4 also reduced the percentage of AxPE (conc.1: 1.62 and Conc.2: 3.303) and PI/AxPE fluorescence (conc.1: 6.81 and Conc.2: 7.84) compared to I/R group (5.06%) and (11.03 %) respectively (figure 14).

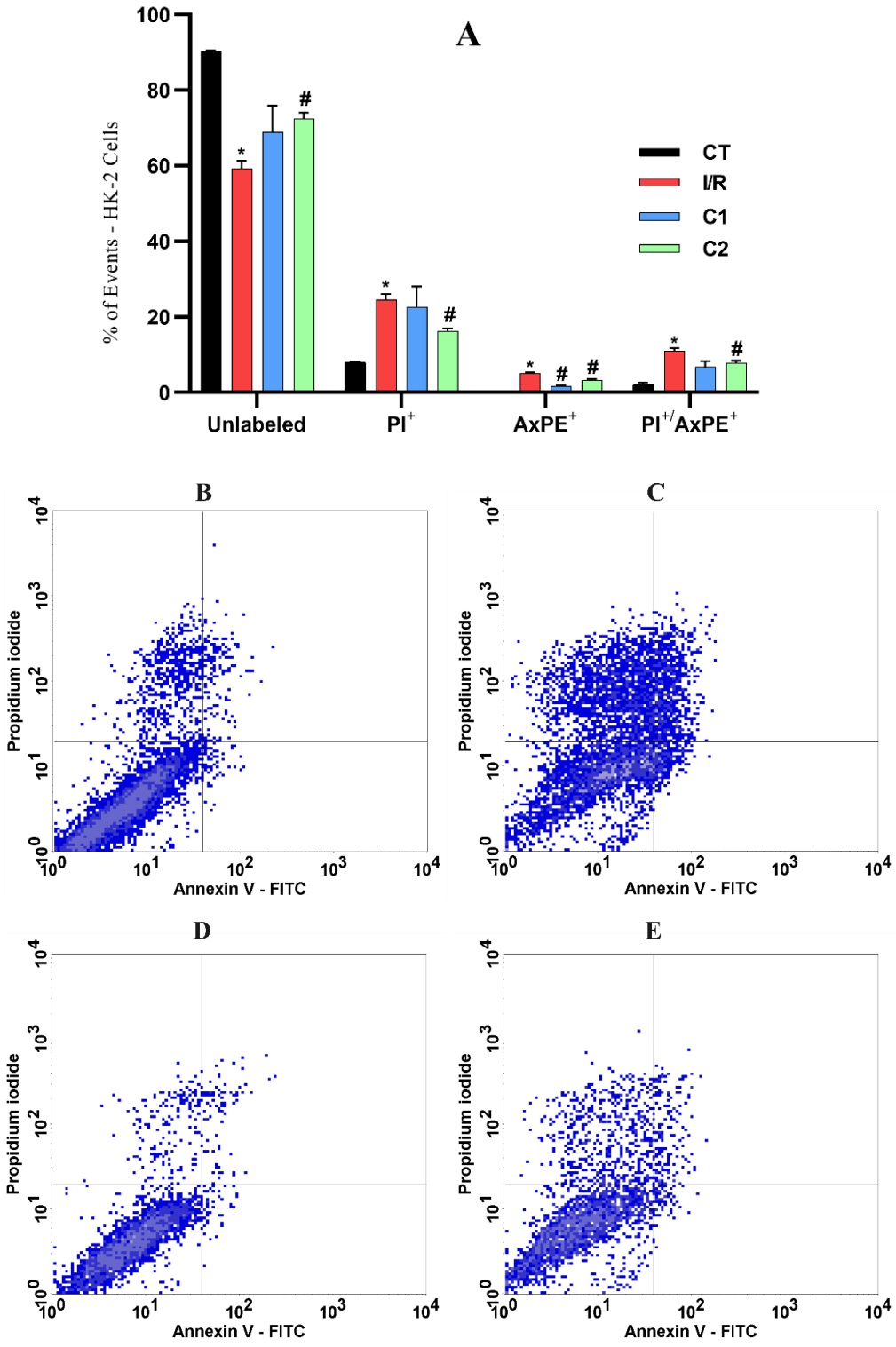

Figure 14: Cell death events assay (Necrosis and Apoptosis), necrotic cells were labeled propidium iodide (PI) and apoptotic cells are labeled with AxPE. (A) Statistical analysis and percent of events in HK-2 cells (B-E) dot plot representation of events in HK-2 cells of control (CT), ischemic reoxygenation exposed (I/R), C1 (31.5 µM) and C2 (15.6 µM) concentrations of chalcone molecule. Results are presented as mean ± S.E.M. $^{*}P < 0.05$ vs. control. #P < 0.05 vs ischemic reoxygenation exposed groups.**Source**: Research data

5.3.2. Analysis of cytoplasmic reactive oxygen species (ROS) generation

To analyze the main causes of apoptosis and necrosis, we examined ROS generation, as previously discussed. We have detailed these cell death events in the above assay. To assess this primary factor in cell death events, we conducted cytoplasmic ROS generation analysis using the DCF-DA for cells staining followed by flow cytometry. HK-2 cells that were exposed to I/R showed significant increase in generation of cytoplasmic ROS compared to control. However, the cells treated with two different concentrations (31.25 and 15.62 μM) of chalcone A4 cells, among which chalcone A4 (15.62 μM) have significantly decreased the DCFH-DA fluorescence (1.26 ± 0.03) compared to I/R group of cells (1.8 ± 0.25) receiving no treatment. The figure 15 and table 1 demonstrate the evaluation of cytoplasmic ROS generation in different group of cells, the effect of chalcone molecule at concentration 15.62 μM in this regard was found significant compared to I/R group of cells.

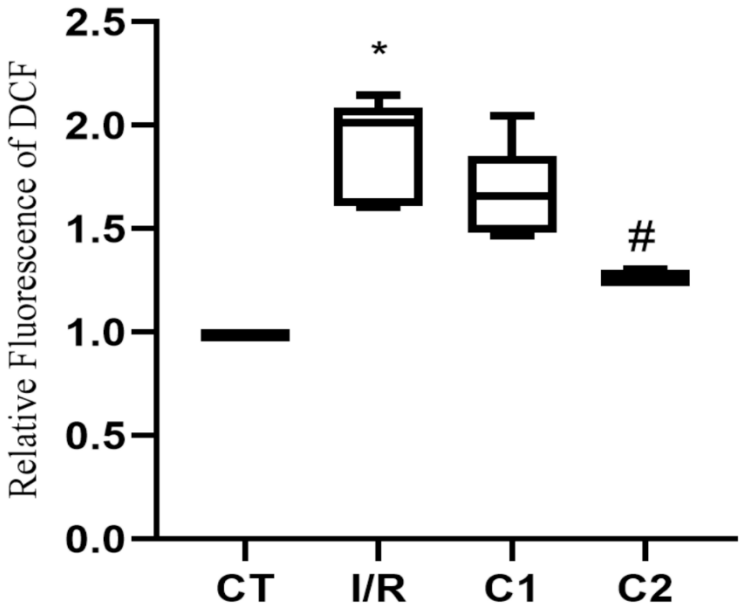

Figure 15: Cytoplasmic reactive oxygen species (ROS) generation in HK-2 Cells analyzed through flow cytometer via DCFH-DA assay. Analysis of relative DCF fluorescence in different groups of HK-2 Cells. HK-2 cells of control (CT), ischemic reoxygenation exposed (I/R), C1 (31.5 μM) and C2 (15.6 μM) concentrations of chalcone A4. Results are expressed as fluorescence ratio relative to control ± S.E.M *p < 0.05 vs control, #p < 0.05 vs ischemia reoxygenation group.**Source:** Research data

5.3.3. Mitochondrial transmembrane potential (ΔΨm) analysis

Mitochondrial transmembrane potential (ΔΨm) plays essential role in biochemical cell regulation and cell death processes. HK-2 cells exposed to I/R has demonstrated significant decrease in Rho123 fluorescence (0.31 ± 0.03) compared to control group (0.92 ± 0.05) of cells indicating loss of mitochondrial function. However, the cells treated with two different concentrations (31.25 and 15.62 µM) of chalcone A4 has increased the relative Rho 123 fluorescence. The increase in Rho 123 fluorescence was found significant with chalcone A4 treatment at concentration 15.62 µM (0.62 ± 0.02) compared to I/R group of cells. The relative fluorescence analysis of Rho123 in different groups of Hk-2 cells (figure 16 and table 1) are shown below.

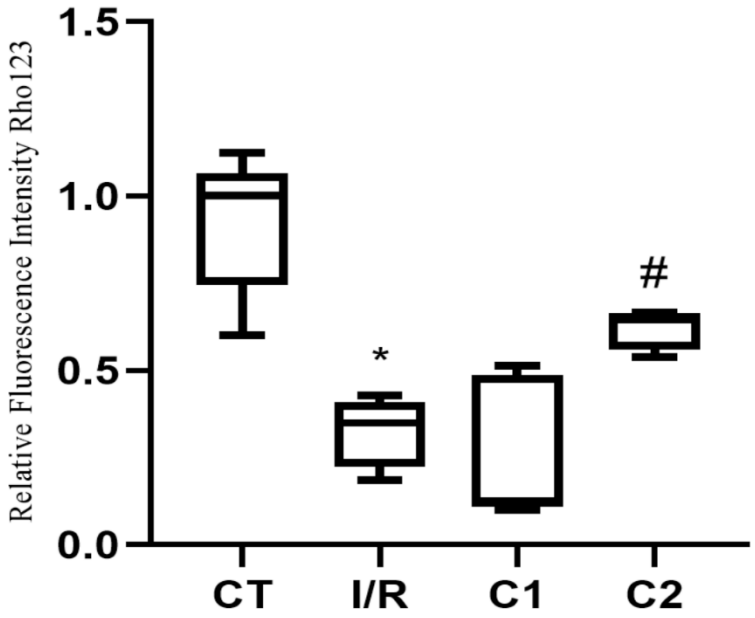

Figure 16: Transmembrane mitochondrial potential analysis in HK-2 Cells, analyzed through flow cytometer via Rho123 assay. Relative fluorescence analysis of Rho123 in different groups of HK-2 Cells. HK-2 cells of control (CT), ischemic reoxygenation exposed (I/R), C1 (31.5 µM) and C2 (15.6 µM) concentrations of chalcone A4. Results are expressed as fluorescence ratio relative to control ± S.E.M *p < 0.05 vs control, #p < 0.05 vs ischemia reoxygenation.

Table 1: Data were expressed as DCF-DA or Rho-123 fluorescence relative to the control ± SEM and analyzed by one-way ANOVA with a post-test (Bonferroni), where *p < 0.05 vs. CT(Control) and #p < 0.05 vs. I/R (Ischemia reoxygenation).

Group	CT	I/R	C1 31.25 µM	C2 15.62 µM
DCF-DA	0.98 ± 0.02	1.8 ± 0.25*	1.66 ± 0.23	1.26 ± 0.03#
Rho-123	0.92 ± 0.18	0.31 ± 0.09*	0.26 ± 0.19	0.62 ± 0.05#

5.4. Oxidative stress analysis

5.4.1. Thiobarbituric acid reactive substances (TBARS) assay

A series of events occurs when cells experience ischemia, and are then reperfused. This process results in oxidative stress and cellular damage. The Thiobarbituric Acid Reactive Substances (TBARS) test, which quantifies lipid peroxidation products, is a prominent indicator of this oxidative damage. This section briefly describes how ischemia-reperfusion affects TBARS levels and offers important information on how much lipid peroxidation occurs in this situation.

The I/R group of cells showed increased concentration of TBARS compared to cells of control group, confirming increase oxidative stress. Treatment with chalcone A4 at both concentration 31.25 and 15.62 µM has significantly reduced TBARS level compared to I/R group of cells. The figure 17 below display TBARS levels in µg/mg of proteins.

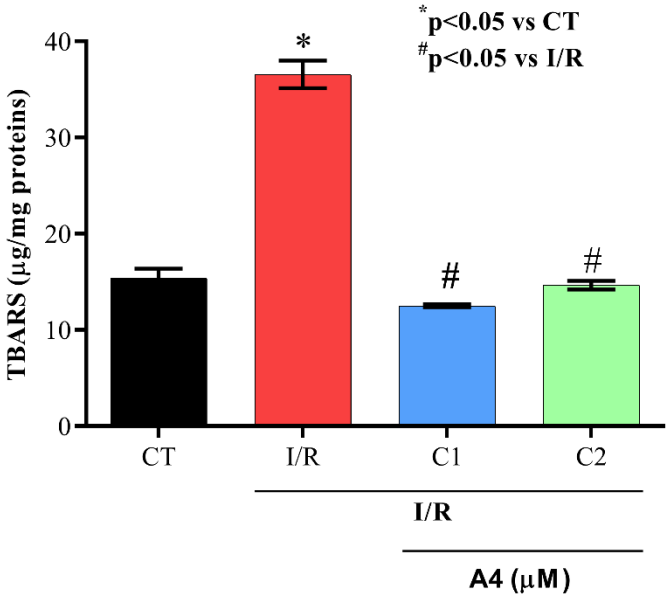

Figure 17: TBARS (Thiobarbituric acid reactive substances) level in different group of HK-2 cells.HK-2 cells of control (CT), ischemic reoxygenation exposed (I/R), C1 (31.5 µM) and C2 (15.6 µM) concentrations of chalcone A4. TBARS level in µg/mg proteins. where *p < 0.05 vs. CT(Control) and #p < 0.05 vs. I/R (Ischemia reoxygenation).

5.4.2. Glutathione (GSH) analysis

Similarly, glutathione (GSH) a thiol-containing tripeptide level was measured, which is an important antioxidant playing its role against xenobiotics, reactive oxygen species (ROS) and other free radicals (Pastore et al., 2003). The I/R induction in HK-2 cells leads to diminished level of GSH compared to normal control group, while treatment with chalcone molecule reversed this phenomenon and increased the level of GSH significantly both at concentration 31.25 and 15.62 µM respectively. The following figures present GSH levels, both in µg/mg of proteins and as a percentage (figure 18).

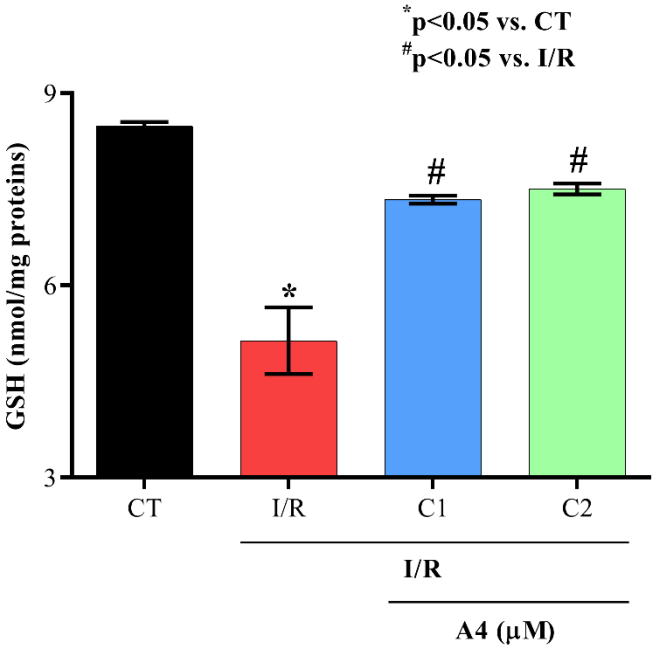

Figure 18: Variation in Glutathione (GSH) levels among HK-2 cell groups.HK-2 cells of control (CT), ischemic reoxygenation exposed (I/R), C1 (31.5 µM) and C2 (15.6 µM) concentrations of chalcone A4. GSH levels measured in µg/mg of proteins. Statistical significance is indicated by *p < 0.05 concerning the Control (CT) group and #p < 0.05 compared to Ischemia-Reoxygenation exposed (I/R) group.**Source:** Research data

5.4.3. Superoxide dismutase (SOD) activity analysis

An important component of the antioxidant defense system, superoxide dismutase (SOD) is a key enzyme in reducing the consequences of ischemia and reperfusion damage. As such, it presents a fascinating target for pharmacological therapies. SOD plays essential role in inactivation of ROS by dismutation of superoxide ($O^{-}2$) free radical into free oxygen (O2) and hydrogen peroxide (H_2O_2). The I/R group of HK-2 cells showed increase level of O^{-}_2 free radical indicating diminished activity of SOD, the cells treated with chalone A4 have significantly decreased O^{-}_2 free radical level at both concentrations (31.25 and 15.62 µM) compared to I/R group (figure 19).

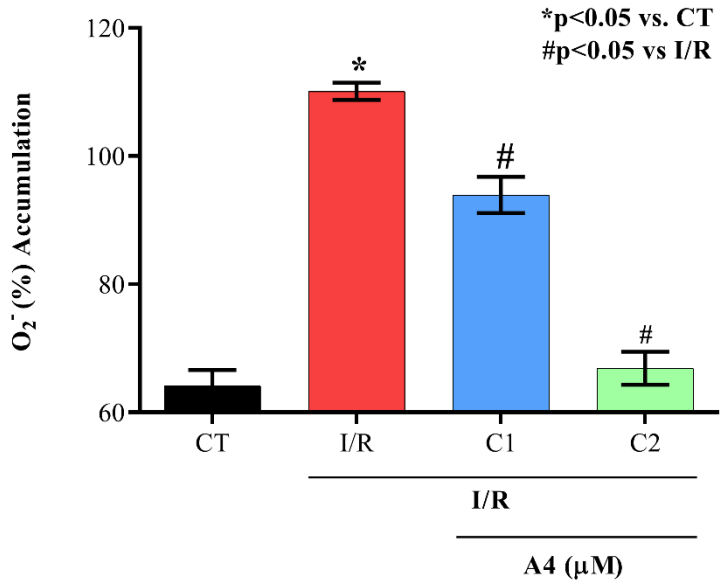

Figure 19: Differences in superoxide dismutase levels within separate HK-2 cell groups are highlighted. HK-2 cells of control (CT), ischemic reoxygenation exposed (I/R), C1 (31.5 µM) and C2 (15.6 µM) concentrations of chalcone A4. Significance is denoted by *p < 0.05 when compared to the Control (CT) group and #p < 0.05 in relation to Ischemia-Reperfusion (I/R).**Source**: Research data

5.5.Scanning electron microscopy

The qualitative alterations happening at the cellular level were observed successfully using the Scanning Electron Microscopy (SEM) method. The images of different groups of HK-2 cells were captured which have revealed various morphological aspects. HK-2 cells exposed to I/R were severely affected indicating cellular damage.

The control group of HK-2 cells shows intact cytoplasm and clear adhesion of cells without any damage to cellular structure while HK-2 cells exposed to I/R shows significant amount of cellular damage with cytoplasm fragmentation and apoptotic blebbing. HK-2 cells treated with chalcone A4 have revert these damages induced by ischemia reoxygenation injury as shown in figure 20.

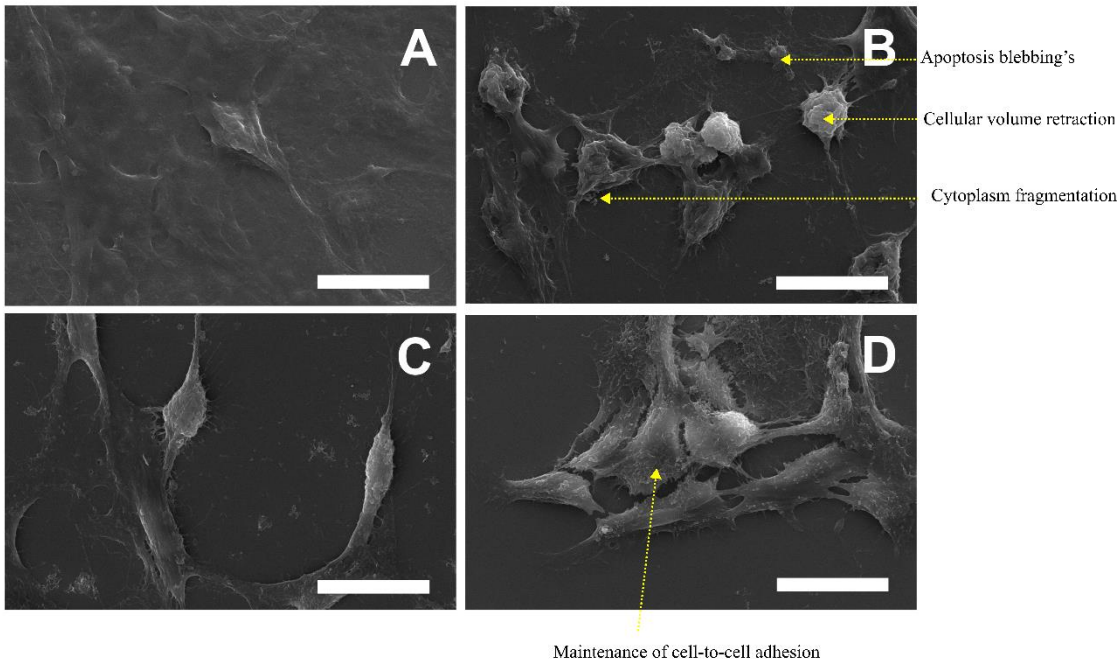

Figure 20: Scanning electron microscope images of different groups of HK-2 cells, 10 µm/5000 x magnification (A) showing HK-2 Cells of control group receiving no treatment, presenting intact structure with uniform cytoplasm and normal morphology (B) HK-2 cell exposed to ischemia reoxygenation receiving no treatment showing apoptosis blebbing with cytoplasm fragmentation (C) HK-2 cells exposed to ischemia reperfusion receiving 31.2 µM of chalcone A4 treatment showing decrease in apoptosis (D) HK-2 cells exposed to ischemia reperfusion receiving 15.6 µM of chalcone A4 treatment showing healing of cytoplasmic fragmentation and decrease apoptosis.

5.6. Molecular docking; inhibition of NADPH oxidase enzyme

To better understand the protection mechanism against I/R-related damage, the inhibition of the NADPH oxidase enzyme was investigated using molecular docking methodology to analyze the molecular interaction of chalcone A4 with the enzyme *in silico*.

Chalcone A4 interacted with the SH3A domain, contained in the p47phox site in the NOX4 isoform, the same site of coupling of the enzymatic inhibitor apocynin. Figure 21 shows the binding site of chalcone A4 and inhibitor, presenting as the most significant interactions. The binding score obtained from chalcone A4 interaction with NOX4 subunit was recorded as -6.7 kcal/mol, suggesting stronger binding interaction and the active nature of chalcone A4. Moreover, chalcone A4 demonstrated four hydrophobic interactions with the residues present in the catalytic site of the enzyme: Leu 177A (3.79 Å), Val 183A (3.87 Å) and Trp 194A (3.94 and 3.98 Å), in addition to three hydrophobic interactions with residues Tyr 161A (3.54 Å), Tyr 167A (3.60 Å), Glu 174A (3.54 Å) and a strong hydrogen bond (Imberty et al., 1991) with residue Cys 196A (2.52 Å), (Table 2)

signifying that these interactions with chalcone A4 may interfere with the activity of the NADPH-oxidase enzyme.

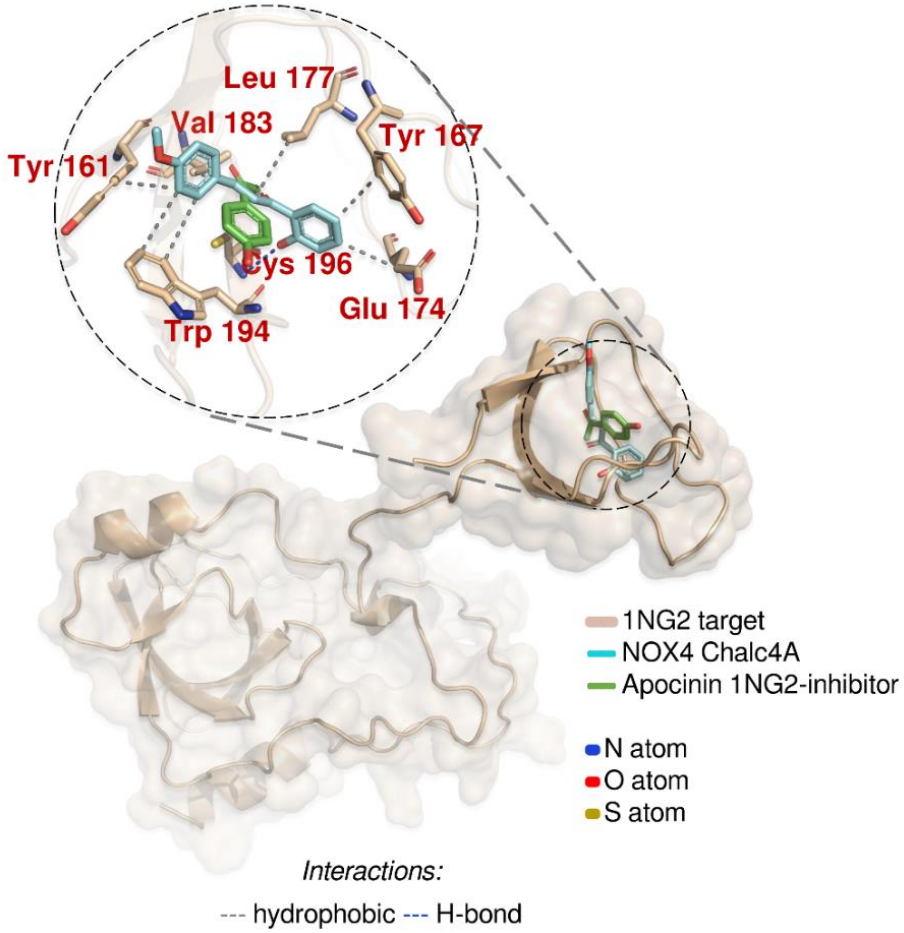

Figure 21: Representation of the molecular coupling of chalcone A4 and the apocynin inhibitor with the NOX4 isoform of the NADPH oxidase enzyme and map of interactions between the ligands and the amino acid residues of the p47phox site present in the NOX4 isoform.**Source**: Research data

Table 2: Interactions of Chalcone A4 and NOX4 subunit of NADPH oxidase.

NOX4/Ligand	Affinity Energy (kcal/mol)	RMSD (Å)	Residue	Interaction	Distance (Å)
Chalcone A4	-6.7	1.95	Tyr-161A	Hydrophobic	3.54
			Tyr-167A	Hydrophobic	3.60
			Glu-174A	Hydrophobic	3.57
			Leu-177A	Hydrophobic	3.79
			Val-183A	Hydrophobic	3.87
			Trp-194A	Hydrophobic	3.98
			Trp-194A	Hydrophobic	3.94
			Cys-196A	H-Bond	2.52

6. Discussion

Ischemia reperfusion injury (I/R) is a phenomenon involving two phases ischemia and reperfusion. Ischemia predominantly cause hypoxia and hypoperfusion of tissues or cells as observed in numerous clinical conditions such as during organ transplantation, sepsis, acute kidney injury and acute coronary syndrome (WU; YIANG; LIAO; TSAI *et al.*, 2018). However, the structural and functional changes in I/R become apparent after restoration of blood flow, which is the reperfusion phase (SOARES; LOSADA; JORDANI; ÉVORA *et al.*, 2019).

The hypoxia and hypoperfusion due to ischemia results in dysfunction of electron transport chain causing decrease production of adenosine triphosphate (ATP) in mitochondria. The decease level of ATP induces anaerobic metabolism along with other dysfunctions such as sodium-potassium and calcium pumps, decrease in ATP and antioxidants levels in cells. These changes lead to increase osmolarity and decrease cellular pH resulting in diminished enzyme function (ORNELLAS; ORNELLAS; MARTINI; CASTIGLIONE *et al.*, 2017). The reperfusion phase cause increased generation of ROS and nitric oxide (NO) levels due to diminished levels of antioxidant enzymes. These discrepancies create an oxidative stressful environment activating local and systemic inflammatory responses (ALI; LIMA SAMPAIO; KHAN; JEANDET *et al.*, 2022; ÁVILA; LÍBANO; ROJAS; RODRIGO, 2019).

Moreover, NOX4 a member of NADPH oxidase family contributes significantly during I/R induced AKI pathogenesis by increasing oxidative stress and activating inflammatory cascades. NOX4 is involved in tubular cell death during I/R. It also plays important role under hypoxic condition in activation of other cellular pathways such as TGF-β1/Smad, and PI3 kinase/Akt/PKC pathway causing kidney injury (CHO; YU; KANG; JEONG *et al.*, 2019; YANG; WU; WANG; GAO *et al.*, 2018).

The *in-vitro* I/R model applied in this study is well established methodology that creates the same milieu (hypoxia and reoxygenation) as observed in I/R (SAMPAIO; LIMA; SILVA; DE AZEVEDO *et al.*, 2019; WENG; LI; SONG; ZHU *et al.*, 2018). The cell viability of HK-2 cells was drastically affected by I/R induction, which was reversed by chalcone A4 treatment significantly at concentration 31.25 and 15.62 μM. However, other chalcones did not provide any significant protection against I/R. It has been reported in literature that hydroxyl group substitution on ring A of chalcone reduced the cytotoxicity of chalcone. The OH group reduce the cytotoxic effect of chalcone by hydrogen bond formation with conjugated group of carbonyl system causing reduction in their reactivity and ability to form Michael adduct (GO; WU; LIU, 2005; GONZÁLEZ; UPEGUI; RIVAS; ECHEVERRI *et al.*, 2020).

In the present study all chalcones have OH group in ring A, the toxic effect on HK-2 cells may be attributed due to the different substituents on ring B of chalcone. Chalcone A1 with furan ring as ring B failed to provide protection I/R induction in HK-2 cells. The NMR spectrum of chalcone A1 showed high field shift due to α, and β-unsaturation, this shift may be caused by the charge displacement either by benzene ring or the double bond making the carbonyl carbon less electron deficient (DA CUNHA XAVIER; DE ALMEIDA-NETO; ROCHA; FREITAS *et al.*, 2021). Chalcone A2 and A6 have Chlorine and Fluorine on ring B respectively, both groups are powerful electron attracting atoms, which may cause destabilization of chalcone. Similar effect was observed in Magalhaes *et al.,* 2022 study, where chloride substitution on ring B showed enhanced toxic effect against parasite *Trypanosoma Cruzi* (MAGALHAES; GOMES; DE FREITAS; SILVA *et al.*, 2022). The chalone A3 which has only OH group on ring A, failed to demonstrate any protection against I/R exposed HK-2 cells. However, chalcone A4 have shown protective effect against I/R possibly due to electron donating ability of methoxy group on its ring B making the whole molecule more stable and active (GONZÁLEZ; UPEGUI; RIVAS; ECHEVERRI *et al.*, 2020).

The elongation of carbonyl system in chalcones present another promising avenue for development and designing of chalcones with tailored biological activities (AL-RIFAI; MUBARAK, 2021; ZHUANG; ZHANG; SHENG; ZHANG *et al.*, 2017). For instance, the five-carbon unsaturated carbonyl system in cinnamylideneacetophenones plays important role in their biological activities with enhanced efficacy as anticancer, antinociceptive and antimicrobial (LE; KIM LIEN; PHAM; TRAN *et al.*, 2023). Here in case of chalcone A5 which has five carbon unsaturated carbonyl system did not provide any protection against I/R, probably because of increase delocalization of π electrons along the molecule causing a change in its reactivity behavior (LUKOVIC; MITROVIC; ZELEN; ČANOVIC *et al.*, 2018).

During reperfusion phase of I/R, increase generation of ROS reacts with proteins, nucleic acids, carbohydrates and lipids, which leads to cellular death pathways activation including apoptosis and necrosis (JIA; WU; PAN; XU *et al.*, 2019). The increase viability of cells after chalcone A4 treatment shows its antioxidant effect. Chalcone molecule increased the cell viability possibly due to inhibition of ROS generation after I/R. Previously, methoxylated chalcones were synthesized via Claisen-Schmidt condensation reaction, among which 3,4,5-trimethoxychalcones showed anticancer, anti-inflammatory and antioxidant action (BANDGAR; GAWANDE; BODADE; TOTRE *et al.*, 2010). Similarly, in another study 25 chalcones derivatives were synthesized via same method, among these, chalcones with -OCH_3 groups were found potent antioxidant (SIVAKUMAR; PRABHAKAR; DOBLE, 2011).

The increase amount of ROS in I/R impairs mitochondria function which further triggers inflammatory and apoptotic pathways (JIA; WU; PAN; XU *et al.*, 2019). Targeting ROS generation in I/R induced AKI is the main therapeutic intervention to manage AKI (HALL; SCHUH, 2016; SAMPAIO; DA COSTA; MENESES; ARRIETA *et al.*, 2016; ZHAO; WANG; LI; LIU *et al.*, 2021). The main pathways in I/R pathogenesis involved apoptosis and necrosis, which impose significant damage to cellular processes (BONVENTRE; YANG, 2011; KOSIERADZKI; ROWIŃSKI, 2008). In our study, I/R group of cells that received no treatment showed significant amount of apoptosis and necrosis events as evident from propidium iodide (PI) and Annexin V-PE (AxPE) fluorescent intensity. The necrosis and apoptosis events were reduced by chalcone A4 treatment significantly with concentration 15.62 μM. The fluorescent dye PI has the ability to distinguish viable, apoptotic and dead cells. PI stains cells during early apoptosis

using permeability of cell membrane (RICCARDI; NICOLETTI, 2006). Similarly, annexin V also detects early programed cell death by binding with integral component “phosphatidylserine (PS)” of plasma membrane. The PS remain intact in healthy cells, during apoptosis it is translocated to outside region of plasma membrane. Annexin V binds with exposed PS as it has high affinity and selectivity for lipid compounds and make the cell stain (KUPCHO; SHULTZ; HURST; HARTNETT *et al.*, 2019). Both PI and AxPE are well established fluorescent dyes for detecting viable and apoptotic cells (KWON; HWANG; SO; LEE *et al.*, 2010; MIYAKE; CHIKUMI; TAKATA; NAKAMOTO *et al.*, 2012; SAMPAIO; LIMA; SILVA; DE AZEVEDO *et al.*, 2019).

Our results showed that chalcone A4 treatment of I/R induced cells has lowered both PI and AxPE fluorescent intensity showing its antioxidant and protective effect. These results correlate with cell viability assay, where chalcone A4 treatment has increased the cell viability compared to I/R group of cells. Previously, chalcones derivatives have been evaluated against cisplatin induced cytotoxicity in LLC-PK1 kidney cells, where they have shown strong nephroprotective effect due to inhibition of MAPKs-p53-caspase-3 pathway and antioxidant action (LEE; KIM; MOON; LEE *et al.*, 2015). Similarly, in another study chalcone derivatives with oxime functional group has ameliorated cisplatin induced cytotoxicity in LLC-PK1 kidney cells. The chalcone derivatives have significantly improved the cell viability and reduced cisplatin induced apoptosis by inhibiting caspase-3, ERK and JNK phosphorylation (LEE; HYUK LEE; LEE; CHOI *et al.*, 2023).

A growing body of evidence shows that mitochondrial dysfunction plays significant role in the etiology of AKI. Mitochondrial dysfunction results in increase generation of ROS, apoptosis and mitochondrial permeability transition (MPT) pore opening (ISHIMOTO; INAGI, 2015; O’TOOLE, 2014). Mitochondria are densely packed in kidney tubule and their dysfunction are thought as important pathological development in AKI. Therefore, mitochondria is considered as prospective target for the novel AKI treatments interventions (HALL; SCHUH, 2016; LI; HEPOKOSKI; GU; SIMONSON *et al.*, 2021). In our study, chalcone A4 dose dependently reduced the generation of cytoplasmic ROS and mitochondrial transmembrane potential as evident from decrease DCF-DA and Rho123 dye intensity respectively. Studies on molecular role of chalcones specifically in I/R induced AKI models are limited. However, there are number of studies regarding its protective role in other models of AKI. Recently, Li *et al.,*

2022 have evaluated chalcone derivatives in cisplatin injury induced AKI models. The findings of their study showed that chalcone ameliorated AKI by inhibiting necroptosis via blocking RIPK1-RIPK3-mixed-lineage kinase domain-like protein (MLKL) signaling pathway (LI; CHEN; HE; LIU *et al.*, 2022). The RIPK3 pathway has been reported to be involved in activation of numerous downstream pathways in I/R injury pathology (JUN; BENJANUWATTRA; CHATTIPAKORN; CHATTIPAKORN, 2020) including generation of ROS from mitochondria (HITOMI; CHRISTOFFERSON; NG; YAO *et al.*, 2008; ZHAO; JAFFER; EGUCHI; WANG *et al.*, 2015). Similarly, another chalcone glycoside molecule (hydroxysafflor yellow A) showed protective effects against I/R injury induced apoptosis in human kidney HK-2 cells (BAI; ZHAO; CUI; WANG *et al.*, 2018). Likewise, Sayed *et al.,* 2022 have comprehensively elaborated the protective role of chalcones in ischemic organ damage, which involved their actions on multiple pathways including Nrf2/Akt activation and NF-κB/TLR4 suppression (SAYED; GOHAR; ABD-ALHAMEED; HASSANEIN *et al.*, 2022). It has been reported that agents that modulates these pathways have protective effect against oxidative injury (SAYED; HASSANEIN; ALI; OMAR *et al.*, 2021; WANG; CHEN; STERNBERG; CAI, 2008).

The increase oxidative stress due to continuous generation of ROS in I/R injury impacts the permeability of cell membrane causing lipid peroxidation. Lipid peroxidation results in saturated and unsaturated carbonyl products including malonyl dialdehyde (MDA), acrolein and heptanal. (GUÉRAUD; ATALAY; BRESGEN; CIPAK *et al.*, 2010; PISOSCHI; POP; IORDACHE; STANCA *et al.*, 2021). Therefore, lipid peroxidation markers are very important in order to identify the progress of disease and level of oxidative stress. The lipid peroxidation is commonly measure by reaction of MDA with thiobarbituric acid (TBA), often called as thiobarbituric acid reactive substance (TBARS) which is a pink color dimeric compound appear at the end of reaction (GHANI; BARRIL; BEDGOOD; PRENZLER, 2017; PISOSCHI; POP; IORDACHE; STANCA *et al.*, 2021). In our study HK-2 cells treatment with chalcone A4 has significantly reduced the TBARS level compared to I/R group of cells (positive control), showing it antioxidant action.

Similarly, glutathione (GSH) a thiol containing tripeptide plays vital role in redox balance, detoxification of drug metabolites and in regulation of gene expression and apoptosis. The mechanism responsible for such actions of GSH involves neutralization of ROS, hydrogen peroxide, protecting cells from ROS induced cell damage and inhibition

of lipid peroxidation (HANSCHMANN; GODOY; BERNDT; HUDEMANN *et al.*, 2013; JEFFERIES; COSTER; KHALIL; BOT *et al.*, 2003). The diminished level of GSH as a result of I/R were reported in various studies and therefore considered as the main target to reverse I/R cellular damage (ALI; LIMA SAMPAIO; KHAN; JEANDET *et al.*, 2022; CAVALCANTI; SAMPAIO; LIMA; COSTA *et al.*, 2023; SAMPAIO; LIMA; SILVA; DE AZEVEDO *et al.*, 2019; YOON; KANG; LEE; JANG *et al.*, 2008). Chalcone A4 treatment of HK-2 cells significantly increased the GSH level compared to I/R group of cells showing it antioxidant action, which correlates with its effect on TBARS level. Furthermore, chalcone A4 treatment of HK-2 cells also significantly reduced singlet oxygen radical (O_2-) accumulation compared to I/R group of cells. Superoxide dismutase (SOD) catalytically coverts O_2- into H_2O_2 and molecular oxygen (O_2), thus protecting cells from harmful effects of free radical. Therefore, SOD is considered as first line of powerful antioxidant enzyme inside cell (IGHODARO; AKINLOYE, 2018). The effect of chalcone molecule on SOD corroborates with previous studies e.g., lavender oil (ABOUTALEB; JAMALI; ABOLHASANI; PAZOKI TOROUDI, 2019) and simvastatin (ZHANG; RONG; FENG; ZHAO *et al.*, 2017) increased the level of SOD causing decrease in oxidative stress resulting in amelioration of I/R induced AKI.

The chalcone A4 treatment at both concentrations (C1 and C2) were able to provide protection against I/R injury in TBARS, GSH and SOD analysis. Nevertheless, this protective effect was not manifested in flow cytometry analysis. The reason for this inconsistency may be attributed to the possibility of multifaceted mechanism of chalcone A4, which may involve pathways beyond the sole mechanism of inhibiting generation of ROS. Antioxidants play important role in preventing oxidative damage and scavenging free radicals, however current research shows that they have limited efficacy in reversing an already established oxidative damage (FORMAN; ZHANG, 2021). This emphasizes how intricately antioxidant defenses interact with the processes that underlie cellular damage and repair in context to I/R injury induced AKI.

The results of SEM imaging further strengthen our previous findings. The cells exposed to I/R shows intense apoptosis blebbing's, and cytoplasm fragmentations. Chalcone A4 treatment has reverse these changes and protected the cells from adverse events of I/R. Numerous *in vivo* studies have shown that I/R induce various morphological changes in kidney cells including tubular dilation, tubular necrosis, cells shrinkage, loos of cells differentiation, glomerular necrosis and cellular atrophy (LAN;

GENG; SINGHA; SAIKUMAR *et al.*, 2016; SENTURK; KABAY; BAYRAMOGLU; OZDEN *et al.*, 2008). The SEM analysis corroborates with these studies and provide another evidence of I/R induce cell damage and morphological alterations.

The results of the *in-silico* study suggest that chalcone A4 is capable of interacting with the cytosolic subunit SH3 domain in p47phox subunit of the NOX4. Both p47phox and p67phox have been known to possess two Src homology 3 (SH3) domains (MANEA, 2010). The central amino acid sequence of p47phox consist of two SH3 domain; SH3A and SH3B (EL-BENNA; DANG; GOUGEROT-POCIDALO; MARIE *et al.*, 2009). The SH3A domain is crucial for protein-protein interactions that are involved in assembly and activation of NADPH oxidase (SOLBAK; ZANG; NARAYANAN; HØJ *et al.*, 2020).

The p47phox subunit of NOX4 is involved in pathogenesis of various diseases including AKI, atherosclerosis and neurodegenerative, primarily through generation of ROS (HOLTERMAN; READ; KENNEDY, 2014; JI; SHI; LIU; HAN *et al.*, 2023). Upregulation of NOX4 is associated with increased renal oxidative stress via increase generation of ROS (LI; ZHANG; WANG; JIANG *et al.*, 2021), as evidenced in diabatic nephropathy where it causes podocyte injury (SEDEEK; NASRALLAH; TOUYZ; HÉBERT, 2013). Similarly, in cisplatin induced AKI, NOX4 plays important role in cell stress responses and programed cell death causing kidney injury (KHODO; DIZIN; SOSSAUER; SZÁNTÓ *et al.*, 2012). This implies the significance of the interactions between chalcone A4 and cytosolic subunit p47phox of NOX4, suggesting its potential to inhibit the generation of ROS and offer protection against I/R induced AKI.

The lower bonding score (-6.7 kcal/mol), several hydrophobic interactions and hydrogen bond interaction with cys-196A (Table 1) of chalcone A4 and NOX4, contribute to overall binding energy, strength of interaction, stability and receptor flexibility (JIANG; KANG; SONG; HUANG *et al.*, 2013). Molecular docking analysis shows that the chalcone A4 has inhibitory activity against Nox4 subunit of NADPH oxidase, implying chalcone A4 as a lead molecule in the development of novel medications for treating I/R induce AKI.

Conclusion

The results of our study demonstrated that Chalcone A4 ((E)-1-(2-hydroxyphenyl)-3-(4-methoxyphenyl)-prop-2-en-1-one) has protected the ischemia reperfusion induced cellular injury via multi actions by increasing cellular viability, reducing apoptosis, generation of cytoplasmic reactive oxygen species and mitochondrial transmembrane potential. Moreover, chalcone A4 has significantly reduced malonaldehyde level as evident from TBARS assay, increased the cellular glutathione and sodium dismutase level, indicating its potent antioxidant effect on cellular antioxidants system. The scanning electron microscopes imaging and molecular docking analysis have further supported the above results showcasing the protective effect of chalcone A4 molecule on HK-2 cells. These results showed that Chalcone A4 possess potential protective characteristics against ischemia reperfusion induced cellular damage offering itself as good candidate for future drug design to ameliorate ischemia reperfusion induce acute kidney injury.

The other five chalcone derivatives failed to provide significant protection against I/R which may link to their structural characteristics against this injury model. Additional modification may enhance their efficacy and selectivity against I/R induced AKI. Therefore, it emphasizes the need to further research the structural activity relationship of these chalcones to identify the important features that may contribute to protective effect against I/R induced AKI. The chalcone A4 efficacy can be further validated by analyzing it effect in animal models of I/R induced AKI. Additional evaluations such as in genetic knockout models, with pharmacological inhibitors or combining its effect with other therapeutic agents (anti-inflammatory or cell signaling modulators) will elucidate it role in I/R induced AKI.

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