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An investigation into kojic acid-associated mitochondrial toxicity and inflammation in melanoma cells (SK-MEL-1)

By

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DECLARATION

I, **Kimera Tamzin Suthiram**, declare as follows:

1. That the work described in this thesis has not been submitted to UKZN or other tertiary institutions for purposes of obtaining an academic qualification, whether by myself or any other party. Where a colleague has indeed prepared a thesis based on related work essentially derived from the same project, this must be stated here, accompanied by the name, the degree for which submitted, the University, the year submitted (or in preparation) and a concise description of the work covered by that thesis such that the examiner can be assured that a single body of work is not being used to justify more than one degree.

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4. **Signed**: **Date:** 14/12/2023

DEDICATION

To my parents, Ramraj and Lilian Suthiram for making my dreams possible.

"For the mountains may move and the hills disappear, but even then, my faithful love for you will remain. My covenant of blessing will never be broken, says the Lord, who has mercy on you"- Isaiah 54:10

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TABLE OF CONTENTS

LIST OF FIGURES viii
LIST OF TABLES x
ABBREVIATIONS xi

ABSTRACT 1

CHAPTER 1 2
1. INTRODUCTION 2
1.1. Hypothesis 5
1.2. Aim 5
1.3. Objectives 6
1.4. Experimental study design 6
1.5. Ethical approval 7
2. LITERATURE REVIEW 8
2.1. The skin 8
2.1.1 Skin structure and function 8
2.1.2 Melanocytes 8
2.1.3. Skin diseases 9
2.2. Kojic acid (KA) 9
2.2.1. Chemical structure and properties 10
2.2.2. Regulations on kojic acid usage 11
2.2.3. Kojic acid inhibits melanogenesis 11
2.2.4. Additional effects of kojic acid 13
2.3. Mitochondrion 16
2.3.1. Mitochondrial function 16
2.3.2. Oxidative stress 16
2.3.3. Master regulators of redox homeostasis 17
2.3.4. Antioxidant responses- Nrf-2 activation 17
2.4. Mitochondrial homeostasis 17
2.4.1. LON proteases 18

2.4.2. Sirtuins 18
2.5. Cell signalling pathways 20
2.5.1. Protein kinase signalling 20
2.5.2. Role of cell signalling in Nrf-2 regulation 23
2.6. Inflammation 24
2.6.1. NFκB inflammatory pathway 24
2.6.2. Inflammasome 25
2.6.3. Inflammation and skin 26
2.7. References 27

CHAPTER 2 46
Exploring the biochemical effects of kojic acid and its implications in humans 46

CHAPTER 3 67
Kojic acid induces oxidative, protein and mitochondrial stress in human melanoma (SK-MEL-1) cells 68

CHAPTER 4 85
Kojic acid induces inflammatory responses independent of the Akt and MAPK signalling pathways 85

CHAPTER 5 106
5.1. CONCLUSION 106
5.2. LIMITATIONS AND RECOMMENDATIONS 108

Addendum 111
Addendum A 111
Addendum B: Guidelines for thesis submission 122
Addendum C: Ethical approval letter 123
Addendum D: Supplementary information 124

LIST OF FIGURES

CHAPTER 1

Figure 1.1: Applications that exploit kojic acid attributes (Prepared by author) 10
Figure 1.2: The chemical structure of KA (Prepared by author) 11
Figure 1.3: Overview of tyrosinase inhibition by kojic acid in melanin production. Chemical structures were drawn using PubChem Sketcher Version 2.4 12
Figure 1.4: The effect of KA in *in vitro* cancer cells (Prepared by author) 13
Figure 1.5: The effect of KA on oxidative stress markers (Prepared by author) 14
Figure 1.6: Structure of Sirtuin proteins found in mammals (Yamamoto et al., 2007) 19
Figure 1.7: The classical three-step cascade of reactions employed to activate ERK1/2 (Kong et al., 2019) 22
Figure 1.8: Schematic illustration of the role of ROS and signalling pathways in the KEAP-1 independent regulation of Nrf-2 (Prepared by author) 24
Figure 1.9: The process of priming and activation of the NLRP3 inflammasome (Lin et al., 2021) ... 26

CHAPTER 2

Figure 2.1: Applications that exploit kojic acid attributes 50
Figure 2.2: Overview of tyrosinase inhibition by kojic acid in melanin production. Chemical structures were drawn using PubChem Sketcher Version 2.4. 52
Figure 2.3: Summary of microbial applications of kojic acid. 57

CHAPTER 3

Figure 3.1: Mitochondrial output was assessed over a 24 h period; a) KA significantly increased cell viability and b) Intracellular ATP was significantly depleted in KA-treated cells. Results are represented as mean fold-change ± SD (n = 3), where ***p < 0.001, **p < 0.01 and *p < 0.05. 74
Figure 3.2: MDA levels were significantly increased in KA-treated cells following a 24 h treatment. Results are represented as mean fold-change ± SD (n = 3), where ***p < 0.001 and **p < 0.01..... 75
Figure 3.3: Nrf-2 expression was significantly increased in KA-treated cells following a 24 h treatment. Results are represented as mean fold-change ± SD (n = 3), where ***p < 0.001 and **p < 0.01..... 75
Figure 3.4: Protein carbonyl levels were significantly increased in KA-treated cells following a 24 h treatment. Results are represented as mean fold-change ± SD (n = 3), where ***p < 0.001 and *p < 0.05. 76
Figure 3.5: KA altered responses to protein damage in KA-treated cells following a 24 h treatment; a) LON protease expression was significantly increased and b) Sirtuin 3 expression was significantly decreased. Results are represented as mean fold-change ± SD (n = 3), where **p < 0.01 and *p < 0.05. 76

CHAPTER 4

Figure 4.1: Kojic acid treatment resulted in alterations in Akt and GSK3β expression. Both the total *Akt* gene expression and phospho-Akt protein levels were significantly reduced ($p < 0.0001$). There was a significant decline in phospho-GSK3β protein expression. The findings are presented as mean fold-change ± SD (n = 3) with ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ denoting statistical significance. 91

Figure 4.2: Kojic acid significantly reduced the expression of ERK1/2.This reduction was particularly noteworthy at high treatment concentrations. The findings are displayed as the mean fold-change ± SD (n=3), where *$p < 0.05$ denotes a statistically significant difference and ***$p < 0.001$ indicates a highly significant difference. 92

Figure 4.3: Kojic acid significantly decreased p38 expression. Results are represented as mean fold-change ± SD (n = 3), where ***$p < 0.001$, and *$p < 0.05$ denotes statistical significance. 93

Figure 4.4: There was increased NFκβ protein and gene expression after a 24 h treatment. Additionally, *IκB* gene expression was increased. The results are represented as the mean fold-change ± SD (n = 3), indicating that statistical significance is denoted by **$p < 0.01$. 93

Figure 4.5: Intracellular caspase 1 and NLRP3 expression was increased at all concentrations. *IL-1β* and *IL-6* were increased significantly at the 100 µg/ml concentration. The results were expressed as mean fold-change ± SD (n = 3), where **$p < 0.01$ and *$p < 0.05$ denotes statistical significance. 94

CHAPTER 5

Figure 5.1: The effect of kojic acid on mitochondrial homeostasis markers, protein kinases and inflammation in SK-MEL-1 melanoma cells..... 108

LIST OF TABLES

CHAPTER 1

Table 1.1: Functions of the layers in the skin 8
Table 1.2: The effect of KA on inflammatory markers in different cell lines 14

CHAPTER 2

Table 2.1: Advances in the toxicity profile of KA in different *in vitro* models 53

CHAPTER 4

Table 4.1: Advances in the toxicity profile of KA in different *in vitro* models..... 90

ABBREVIATIONS

ADP- Adenosine diphosphate

Akt- Ak strain transforming

AP-1- Activator protein 1

ARE- Antioxidant-responsive element

ASK-1- Apoptosis signal-regulating kinase-1

ATP- Adenosine triphosphate

bZIP- Basic region leucine zipper

CCL-2 – Chemokine ligand-2

CIR- Cosmetic Ingredient Review

CRP- C-reactive protein

DALY- Disability-adjusted life year

DAMP- Damage associated molecular pattern

DLK1- Dual leucine-zipper-bearing kinase-1

ER- Endoplasmic reticulum

ERK1/2- Extracellular signal-response kinase 1/2

FADD- Fas-associated protein with death domain

FDA- Food and Drug Administration

GBD- Global Burden of Disease

GPx- Glutathione peroxidase

GSK3β- Glycogen synthase kinase 3β

HCEC- Human corneal epithelial cell

IARC- International Agency for Research on Cancer

IFN-β- Interferon-β

ILDS- International League of Dermatological Societies

iNOS- Inducible nitric oxide synthase

JNK- c-Jun N-terminal kinase

KA- Kojic acid

KEAP-1 - Kelch-like-ECH-associated protein-1

MAPK- Mitogen activated protein kinase

MEKK- MAPK/ERK kinase kinase

MITF- Microphthalmia-associated transcription factor

mTOR- Mammalian target of rapamycin

MyD88- Myeloid differentiation primary response 88

NAD^+- Nicotinamide adenine dinucleotide

NEMO- NFκB essential modulator

NFκB- Nuclear factor kappa-light-chain-enhancer of activated B cells

NLRP3- NLR family pyrin domain containing 3

NO- Nitric oxide

NOD2- Nucleotide-binding oligomerization domain 2

Nrf-2 - Nuclear factor erythroid 2-related factor-2

p53- Tumour suppressor protein 53

PAMP- Pathogen associated molecular pattern

PDK1- Phosphoinositide dependent kinase 1

PGC-1α - Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PI_3K- Phosphoinositide 3 kinase

PIP_2 - Phosphatidylinositol (3,4)-bisphosphate

PIP_3- Phosphatidylinositol (3,4,5)-trisphosphate

PRR- Pattern recognition receptors

Prx- Peroxiredoxins

PTEN- Phosphatase and tensin homolog

RAGEs- Receptors for advanced glycation end products

ROS- Reactive oxygen species

SCCP- Scientific Committee on Consumer Products

TAK1- Transforming growth factor β-activated kinase 1

TLR- Toll-like receptors

TNF- Tumour necrosis factor

TRIF- TIR domain-containing adaptor-inducing interferon-β

UV- Ultra-violet

WHO- World Health Organisation

α-MSH- Alpha melanocyte-stimulating hormone

β-TrCP- β-Transducing repeat-containing protein

ABSTRACT

Kojic acid (KA), 5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one, is used in agriculture, food, and cosmetics. KA is known to have antimicrobial, antifungal, antioxidant, and anti-inflammatory properties. The cosmetic industry's increasing interest in KA is due to its ability to inhibit tyrosinase activity resulting in skin lightening. The mitochondria play a key role in maintaining homeostasis and ensuring efficient melanin production. Therefore, mitochondrial dysfunction has severe effects on the skin. This study investigates mitochondrial stress, antioxidant responses, protein kinase signalling and inflammation in human melanoma (SK-MEL-1) cells.

The mitochondria are important in processing metabolites and supplying the cell with energy in the form of ATP. KA interacts with key mitochondrial homeostasis proteins. Our results found an increase in macromolecule damage specifically lipid peroxidation and protein oxidation. Due to oxidative conditions, increased Nrf2 expression was observed. LON protease is ATP-dependent and regulated by Sirtuin 3 expression. Mitochondrial function was affected illustrated by decreased ATP production leading to decreased LON protease and Sirtuin 3 protein expression.

Following increased oxidative stress, KA suppressed the expression of protein kinases but increased inflammatory mediators. There was decreased expression of phospho-Akt, *Akt*, phospho-GSK3β, p38 and ERK1/2. The mediation of the NLRP3 inflammasome involves priming and activation. At concentrations with high proliferation, NFκB gene and protein expression was activated. The protein kinase signalling pathways are known as mediators of inflammation; however, protein and gene expression of inflammatory mediators was increased following KA treatment. The inflammasome was subsequently activated as shown by an increase in intracellular caspase 1 levels as well as *NLRP3, IL-β* and *IL-6* expression.

KA induced mitochondrial stress and suppressed mitochondrial homeostasis proteins. The increased Nrf2 expression could have further downregulated LON protease expression and increased macromolecule damage. Oxidative conditions could have activated the inflammasome pathway independent of protein kinase signalling. In conclusion, KA displayed mitochondrial toxicity following acute exposure by suppressing mitochondrial homeostasis, protein kinase pathways and initiating inflammation.

CHAPTER 1

1. INTRODUCTION

The skin serves as the body's largest organ, acting as a defence barrier against chemical and physical harm (Blume-Peytavi et al., 2016). The International League of Dermatological Societies (ILDS) has concluded that ageing skin is a significant challenge to skin health (Blume-Peytavi et al., 2016). As the skin ages, it becomes more prone to dysregulation, leading to various skin conditions and an increased risk of developing skin cancers (Farage et al., 2009). Skin ageing is time-dependent and occurs because of intrinsic (natural) and extrinsic (environmental influences) factors (Blume-Peytavi et al., 2016).

According to the World Health Organization (WHO), skin-lightening products are most popular among African and Asian populations (Chib et al., 2023). Kojic acid (KA) is a popular ingredient in skin-lightening creams, with an estimated market of $31.2 billion by 2024 (Chib et al., 2023, Felipe et al., 2023). Fungi belonging to the genera *Aspergillus* and *Penicillium* produce KA through the biosynthesis of γ-pyrone from sugar (Parrish et al., 1966, Mohamad et al., 2010, El-Kady et al., 2014, Phasha et al., 2022, Rasmey and Abdel-Kareem, 2021). Industrial production has been optimized to meet the high demand for KA (Chib et al., 2023, de Caldas Felipe et al., 2023).

KA is a versatile compound used in the medical, food, and cosmetic industries. In the cosmetic industry, its weak acid properties and structure enable various applications, including anti-ageing and skin brightening. The KA structure is composed of a γ-pyrone ring, a hydroxymethyl group, and a methyl group. Research studies have shown that a safe concentration of KA is between 0.1% and 2% (Mann et al., 2018, Burnett et al., 2010a). According to the regulation set by the Scientific Committee on Consumer Products (SCCP) of the European Commission, the maximum permissible limit for KA as an ingredient is 1% (SCCP, 2021). For leave-in creams containing KA, the safe dose is 1% (Saeedi et al., 2019). The studies conducted did not find any instances of allergic reactions or sensitivity (Saeedi et al., 2019, Phasha et al., 2022). Moreover, KA has been classified as a group 3 carcinogen by the International Agency for Research on Cancer (IARC). However, there are contrasting regulations by the Food and Drug Administration (FDA), which states that KA should only be used with a prescription. On the other hand, the SCCP found no toxicity associated with KA (Saeedi et al., 2019). Studies have shown that the compound may cause damage to the skin through exposure to light and can also result in contact dermatitis (Saeedi et al., 2019). KA studies on the effects in humans are limited. KA has been used for hyperglycaemia (Singh et al., 2007), cancer (Fang et al., 2015, Kumari et al., 2018, Nakhi et al., 2012), excess proliferation (Peroković et al., 2020), oxidative stress (Niwa and Akamatsu, 1991, El-Metwally et al., 2020), hyperpigmentation (Lajis et al., 2012a), and inflammation (Moon et al., 2001).

KA displays biochemical effects on different cell lines. KA is a known antioxidant (Van Tran et al., 2019, Kumar et al., 2013). To avoid oxidative damage during mycelial degeneration, KA triggers the activation of antioxidant pathways (Zhang et al., 2017). KA has been found to possess free radical scavenging abilities in humans (Lajis et al., 2012a). Liver cells were found to have increased activity of nuclear factor erythroid 2-related factor-2 (Nrf2) following KA exposure (Suthiram et al., 2023), which agrees with the compound's antioxidant potential. Additionally, KA displays anti-inflammatory effects. KA and its derivatives possess chelating properties that activate multiple pathways, resulting in favourable anti-inflammatory effects (Brtko et al., 2004a, Lee et al., 2019b, Li et al., 2021, Zhang et al., 2017, Khan et al., 2021). In terms of the mitochondrial implications of its use, the only study to our knowledge focused on the chemo-sensitising of complex III inhibitors in fungi (Kim et al., 2013a).

There is limited research into the effects of KA in pathways related to mitochondrial stress and inflammation, although many properties have been discovered and utilized in the prevention of hyperpigmentation. Our previous study assessed the antioxidant and inflammatory response in HepG2 liver cells. Following a 24 h treatment, increased Nrf2 and Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) responses were observed (Suthiram et al., 2023)(Addendum A). This study suggests that treatment with KA could lead to inflammation and antioxidant/oxidant imbalances. Studies investigating KA's role in mitochondrial stress, protein kinase signalling, and inflammation are not fully comprehended. In recent years, the use of KA has increased due to hydroquinone being prohibited in some countries and regulators discouraging its use. Therefore, by understanding the effect on key markers associated with skin disease and ageing, we can understand its role in alleviating or exacerbating disease. This is beneficial specifically to populations that use skin-lighteners daily and are prone to skin pathologies due to their use.

Most skin diseases are exasperated by mitochondrial aberrations, oxidative stress, and inflammation. High concentrations of reactive species perturb mitochondrial functioning. High concentrations of reactive oxygen species (ROS) negatively affect cells resulting in oxidative stress (Schieber and Chandel, 2014, Mailloux, 2020). ROS is produced when electrons react with oxygen and escape the electron transport chain (Lennicke and Cochemé, 2021). A balance is required between the production and removal of ROS which is facilitated by antioxidant systems. Nrf2 enables the transcription of key antioxidant proteins involved in maintaining homeostasis. Residues between bound Nrf2 and Kelch-like-ECH-associated protein 1 (KEAP-1) are oxidised to enable the release of Nrf2. This process activates the translocation of Nrf2 to the nucleus, where the transcription of genes for proteins such as manganese superoxide dismutase (MnSOD) takes place.

Sirtuins in mammals are a family of seven members, namely Sirtuins 1-7. They are categorised based on the conserved catalytic core domain they possess (Schwer and Verdin, 2008a, Zhang et al., 2020).

Sirtuins are dependent on the cofactor, nicotinamide adenine dinucleotide (NAD^+), that promotes deacetylation reactions (Someya et al., 2010). Sirtuin 3 eliminates oxidative stress by suppressing ROS production and inhibiting inflammation. Sirtuin 3 activates autophagy resulting in the suppression of the inflammasome (Zhang et al., 2020). Studies illustrate the relationship between Sirtuin 3 and protein kinases, Protein kinase B (Akt) and mitogen-activated protein kinase (MAPK) signalling. Sirtuin 3 protects against liver fibrosis by limiting ROS production and activating glycogen synthase kinase 3β (GSK3β) (Sundaresan et al., 2015). Sirtuin 3 has been found to have beneficial effects against ROS in cardiac tissue through reactions with extracellular signal-regulated kinase 1/2 (ERK1/2) (Fan et al., 2018). Sirtuin 3 deacetylation activity can both increase and decrease GSK3β activity. Furthermore, NFκB binds to the Sirtuin 3 promotor region and enhances its expression. All reactions facilitated by Sirtuin 3 in the mitochondria ensure metabolic stability (Neeli et al., 2020).

Another role of Sirtuin 3 is to deacetylate LON protease resulting in the inhibition of tumorigenesis, therefore, regulating the lysosomal degradation of LON protease (Wu et al., 2023). LON is an ATP-dependent protease that forms part of the mitochondrial protein quality control system. Under conditions of oxidative stress, LON protease degrades misfolded proteins. In this way, LON protease prevents the accumulation of damaged proteins (Hanson and Whiteheart, 2005, Voos and Pollecker, 2020).

Oxidative stress can be detrimental to macromolecules and promote inflammation (Yuan et al., 2022). Excess ROS has been known to activate inflammatory markers such as the NLR family pyrin domain containing 3 (NLRP3) inflammasome (Zhou et al., 2011). Inflammatory mediators can be activated by protein kinases. These protein kinases include Akt and the MAPK signalling pathway. The Akt signalling pathway occurs through a highly conserved multistep process. Activated receptors stimulate bound PI_3K with regulatory proteins or adaptor proteins. The activation of PI_3K leads to the conversion of phosphatidylinositol (3,4)-bisphosphate (PIP_2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). Akt binds to the PIP_3 that is found on the plasma membrane. This process requires phosphoinositide-dependent kinase 1 (PDK1) phosphorylation resulting in partial activation of Akt (Alessi et al., 1997, Hemmings and Restuccia, 2012). Proteins involved in downstream pathways such as the mammalian target of rapamycin (mTOR) fully activate Akt (Sarbassov et al., 2005). Activated Akt performs functions in metabolism, cell growth, cell proliferation, cell survival, angiogenesis, protein synthesis, transcription and apoptosis (Hemmings and Restuccia, 2012).

MAPK signalling is an intracellular pathway involved in several processes such as cell proliferation, cell differentiation, apoptosis, angiogenesis, tumour metastasis and stress responses. MAPK proteins are dually phosphorylated through a cascade of reactions involving MAP3K, MAPKK and MAPK that activate downstream processes (Guo et al., 2020, Plotnikov et al., 2011, Wortzel and Seger, 2011).

Several studies have illustrated the relationship between MAPK proteins and NFκB expression. MAPK proteins include p38, c-Jun N-terminal kinase (JNK) and ERK1/2. MAPK p38 has been found to activate pro-inflammatory mediators (Saha et al., 2007) whereas ERK1/2 has been found to suppress inflammation (Pastore et al., 2005). Some cell lines display ERK1/2 as an initiator of inflammatory responses (Xiao et al., 2002).

Inflammation occurs in response to factors such as infection or damage to cells and tissue (Lawrence, 2009). NFκB responds to stimuli initiated by the ligand binding to receptors such as pattern recognition receptors, tumour necrosis factor (TNF) receptors, cytokine receptors, T-cell receptors and B-cell receptors (Zhang and Sun, 2015). NFκB has two mechanisms of activation, the canonical and non-canonical pathways. The canonical pathway involves the degradation of IκB through the phosphorylation by the IκB kinase (IKK) complex (Oeckinghaus and Ghosh, 2009, Karin and Delhase, 2000). IKK is activated by cytokines, growth factors, stress and microbial components (Israël, 2010). IKK facilitates the phosphorylation and degradation of IκB in the proteasome leading to the nuclear translocation of NFκB. NFκB plays a role in regulating the formation of the NLRP3 inflammasome (Sutterwala et al., 2014).

The control of the NLRP3 inflammasome assembly requires a two-step process of priming and activation. The priming step results in the activation of NFκB signalling. The upregulation of NFκB expression transcribes NLRP3, pro-IL-1β and IL-18 leading to the activation of the inflammasome. Secondary signalling results in the assembly and secretion of mature IL-1β. Mitochondrial damage is a stimulus linked to NLRP3 activation (Liu et al., 2017). Activated NFκB delays the accumulation of the autophagy receptor p62 which binds to mitochondrial poly-ubiquitin chains and negatively regulates the inflammasome. IκB favours autophagy over inflammasome activation (Liu et al., 2017, Zhong et al., 2016, Criollo et al., 2010).

1.1. Hypothesis

It was hypothesised that KA induced mitochondrial stress that will affect protein kinase and inflammatory pathways.

1.2. Aim

The study aimed to investigate the role of KA in antioxidant/oxidant responses and inflammation in SK-MEL-1 cells.

1.3. Objectives

The mitochondrial toxicity and subsequent downstream implications were assessed by measuring cytotoxicity as well as protein and gene expression of mitochondrial stress proteins, protein kinases and inflammatory markers in SK-MEL-1 cells. The specific objectives of the study were as follows:

- KA-induced cytotoxicity was assessed by measuring mitochondrial output (MTT and ATP luminometry) and mitochondrial homeostasis proteins specifically LON protease, Sirtuin 3 and antioxidant response protein Nrf-2. Confirmation of oxidative stress markers used lipid peroxidation, and protein carbonyl assay.

- KA-induced regulation of protein kinase signalling (MAPK and Akt) pathways and inflammation was assessed by measuring intracellular caspase 1 concentrations using luminometry, protein expression (phospho-Akt, phospho-GSK3β, ERK1/2, p38, and NFκB) using Western Blot and gene expression (*Akt, NFκB, Iκβ, NLRP3, IL-1β, IL-6)* using qPCR.

1.4. Experimental study design

Since the SK-MEL-1 cells produces melanin, it is an appropriate skin cell line to use when evaluating the safety and efficacy of KA. While the melanin inhibition mechanism of KA has already been established, we evaluated signalling proteins linked to tyrosinase activity. The cells used in the study is a tumorigenic cell line. The cell line originated from a 29-year-old white, male patient suffering from melanoma disease. This further allowed for the assessment of KA effects on cancer cells and subsequent pathways. Treatment conditions were selected due to applications of skin lighteners occurring once daily (0-500 µg/ml; 24 h). The study utilised methods to assess cell viability and intracellular ATP levels to determine mitochondrial output. This provided insight into concentrations that were suitable for use and lacked cytotoxic potential. These concentrations were used to investigate macromolecule damage with a specific focus on protein oxidation and lipid peroxidation. To further confirm the results, protein and gene expression of key markers involved in cell signalling and inflammation were evaluated.

This thesis is being submitted according to the guidelines set out by the University of KwaZulu-Natal in the form of three manuscripts comprising one review paper and two experimental research papers (Addendum B):

1. A comprehensive literature review was included in Chapter 1 with additional literature pertaining to the thesis. A review paper entitled “Exploring the biochemical effects of kojic acid and its implications in humans” based on the literature review was submitted to *Toxicon* (TOXCON-D-23-00615) and included in Chapter 2.

2. Chapter 3 includes an experimental article focusing on kojic acid's effect on mitochondrial homeostasis in SK-MEL-1 cells that was submitted to the *Journal of Toxicology*.

3. Chapter 4 includes an experimental article focusing on the effect of kojic acid on protein kinase B/Akt and MAPK signalling resulting in the activation of inflammation in SK-MEL-1 cells that was submitted to *Experimental Dermatology* (EXD-23-0879).

1.5. Ethical approval

The study obtained ethical approval from the Biomedical Research Ethics Committee (University of KwaZulu-Natal- protocol reference number: BREC/00005725/2023; Addendum C) for all work performed on SK-MEL-1 cells.

2. LITERATURE REVIEW

2.1. The skin

2.1.1 Skin structure and function

The average adult human skin has a surface area of approximately 2 square meters. The skin is the body's primary barrier against toxins, ultraviolet radiation, pathogens, and physical harm (Amsden and Goosen, 1995). The skin performs vital functions such as preventing fluid loss and regulating body temperature (Burns et al., 2008, Hwa et al., 2011). It comprises three layers: epidermis, dermis, and hypodermis (Orioli and Dellambra, 2018). Each layer protects the body (Table 1.1).

Table 1.1: Functions of the layers in the skin

LAYERS IN THE SKIN		
Epidermis	Dermis	Hypodermis
The epidermis comprises keratinocytes that differentiate to renew the epidermis, while stem cells and transient cells maintain their integrity (Barrandon et al., 2012).	The dermis is a layer of connective tissue composed of fibroblasts that secrete collagen and matrix proteins. Biochemical signalling is essential for stem cell maintenance, healing, and disease prevention in the dermis (Sriram et al., 2015, Murphree, 2017).	The hypodermis consists of an adipose layer that connects the skin to muscles and bones (Orioli and Dellambra, 2018).

The skin undergoes damage from chronological, photo-, and premature ageing. Therefore, the ageing of this vital organ is tightly regulated at the protein and epigenetic level to prevent disease.

2.1.2 Melanocytes

Melanocytes are heterogeneous cells that originate from neural crest cells. The cells produce melanin, a pigment that gives colour to the skin, hair, and eyes. Additionally, these cells perform numerous other functions (Cichorek et al., 2013). The melanocyte life cycle begins with the development of melanoblasts from neural crest cells. Melanoblasts migrate, proliferate, and differentiate into melanocytes. Mature melanocytes synthesize melanin in melanosomes and change to exhibit a dendritic morphology. Melanocytes transport mature melanosomes to keratinocytes and eventually, cell death occurs (Cichorek et al., 2013). Melanocytes secrete several signalling molecules that target keratinocytes and immunological systems. These include proinflammatory cytokines (IL-1, IL-2, IL-3,

IL-6, IL-10, and TNF-α), chemokines (IL-8 and chemokine ligand-2 (CCL2)), transforming growth factor (TGF-β), catecholamines, eicosanoids, serotonin, alpha melanocyte-stimulating hormone (α-MSH), and nitric oxide (NO) (Lu et al., 2002, Chen et al., 2022). IL-1, IL-6, and TNF-α inhibit melanogenesis, whereas eicosanoids and α-MSH increase melanogenesis (Slominski et al., 2004).

2.1.3. Skin diseases

Skin disease is the fourth most common in humans and affects one-third of the world. Skin diseases can have severe psychological, physical and financial effects (Basra and Shahrukh, 2009), however, this field of research is often neglected. The 'burden of disease' describes the loss of health because of illness and injury. This factor was based largely on the disability-adjusted life year (DALY) (Flohr and Hay, 2021). DALY refers to the loss of time due to premature death and disability. Among diseases measured by DALYs using the Global Burden of Disease (GBD), atopic dermatitis ranked 15th and is the highest among skin diseases (Laughter et al., 2021). Acne vulgaris is the most common inflammatory skin disease (Layton et al., 2021). Additionally, the World Health Organisation (WHO) identified psoriasis as a disfiguring disease that imposes a significant social, economic and psychological burden (Iskandar et al., 2021). Oxidative stress and inflammation are common pathways associated with skin disease.

2.2. Kojic acid (KA)

KA is produced by *Aspergillus* and *Penicillium* (Parrish et al., 1966, Mohamad et al., 2010, El-Kady et al., 2014, Phasha et al., 2022, Rasmey and Abdel-Kareem, 2021). There are various applications for KA in the food, cosmetic, pharmaceutical, and agricultural sectors (Figure 1.1). KA is a pesticide and insecticide (Lee et al., 1950, Beard and Walton, 1969, Uher et al., 1994, Kim et al., 2012, Wu et al., 2019). KA is frequently used as a flavour-enhancing ingredient and to preserve food by keeping fruit from discolouring (Chen et al., 1991, Burnett et al., 2010b, Deri et al., 2016). KA is used in Japanese traditional foods and beverages (Kotyzova et al., 2004).

KA is mainly used as a tyrosinase inhibitor in cosmetics (Bentley, 2006, Hasil et al., 2020, Suryadi et al., 2022). The safety of cosmetic items is assessed by evaluating their toxicity. Medical applications exploit KA's ability to inhibit inflammatory, oxidative stress, and cancer (Zilles et al., 2022, Suthiram et al., 2023). Human dysregulation of melanin synthesis is the root cause of many skin disorders. KA has demonstrated the ability to treat skin conditions such as leishmaniasis by lowering parasitic burden (Rodrigues et al., 2014, Saeedi et al., 2023, Brtko, 2022).

Because KA was found in maize and chicken feed and co-existed with aflatoxins (a mycotoxin that is highly hepatotoxic), it was initially categorized as a mycotoxin (Parrish et al., 1966, Souza et al., 2013). Recent studies on KA have led some researchers to conclude that there is minimal risk of mycotoxin

exposure for humans (Bentley, 2006). The reason for KA's inconsistent classification could be due to a lack of studies on various areas of KA's effectiveness on human health.

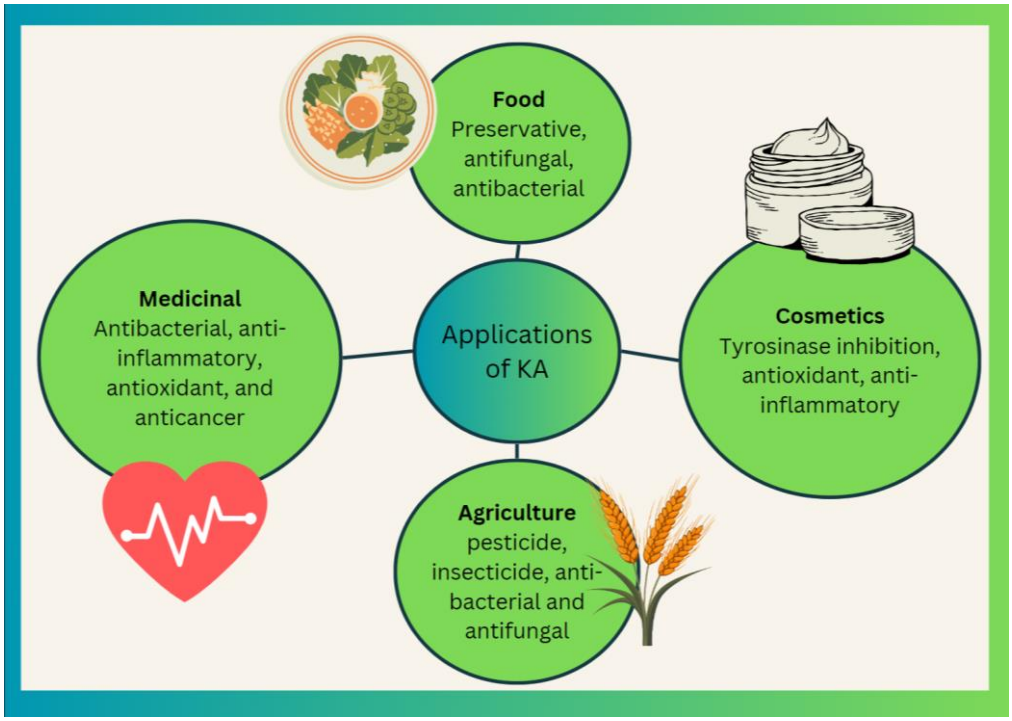

Figure 1.1: Applications that exploit kojic acid attributes (Prepared by author)

2.2.1. Chemical structure and properties

The chemical nomenclature for KA is 5-hydroxy-2-hydroxymethyl-γ-pyranone ($C_6H_6O_4$). Saito (1907) first discovered KA, but Yabuta (1924) only later determined the structure. KA is a weak heterocyclic acid that has hydroxymethyl and hydroxyl groups, respectively, in the C2 and C5 positions (Figure 1.2) (Yabuta, 1924, Beelik, 1956, Ichimoto et al., 1965). Because of the compound structure, metals such as sodium, copper, zinc, calcium, nickel, and cadmium can chelate to produce salts (Beelik, 1956, Coupland and Niehaus, 1987, Saeedi et al., 2019). This characteristic has been used to suppress melanin production.

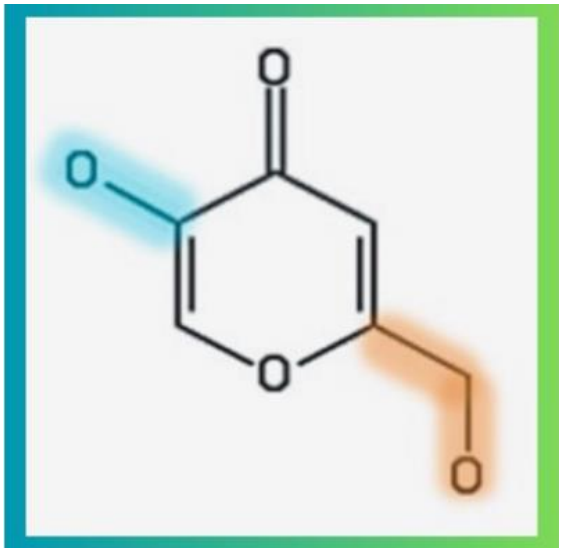

Figure 1.2: The chemical structure of KA (Prepared by author)

2.2.2. Regulations on kojic acid usage

Because of health concerns raised by published research on the relationship between KA and skin sensitivity, the European Commission's SCCP established a maximum concentration of 1% KA in skincare products (SCCP, 2021). Moreover, the Cosmetic Ingredient Review (CIR) found that skin lightening and sensitization cannot occur at doses lower than 1% (Chib et al., 2023).

2.2.3. Kojic acid inhibits melanogenesis

Hormones, genes, and the immune system are among the intrinsic variables that drive melanogenesis; external factors include exposure to chemicals and ultraviolet light (Talebi et al., 2022). Melanin is primarily responsible for shielding the skin and eyes from harm. Melanogenesis occurs in stages. Melanocytes generate melanin, which is formed from tyrosine (Wawrzyk-Bochenek et al., 2023). Melanosomes in the epidermis contain the glycoprotein known as polyphenol oxidase or tyrosinase. Animal skin, eyes, and hair epidermal cells' colour is determined by this enzyme (Pekkarinen et al., 1999). Tyrosinase is a metalloprotein enzyme containing histidine residues that bind copper ions at its active site and is responsible for the biosynthesis of Levodopa (L-DOPA) (Pawelek and Körner, 1982, Mayer, 1986, Cabanes et al., 1994, Gillbro and Olsson, 2011). Tyrosinase catalyses two steps in melanogenesis: (1) hydroxylation of tyrosine to L-DOPA as monophenolase and (2) oxidation of L-DOPA to dopaquinone (Figure 1.3) (Kim et al., 2003). The subsequent biosynthetic steps are non-enzymatic, thus making tyrosinase a key enzyme in the rate-limiting steps. The differing factor between monophenolase and diphenolase activity is determined by the hydroxyl molecule found between the copper ions (Baber et al., 2023).

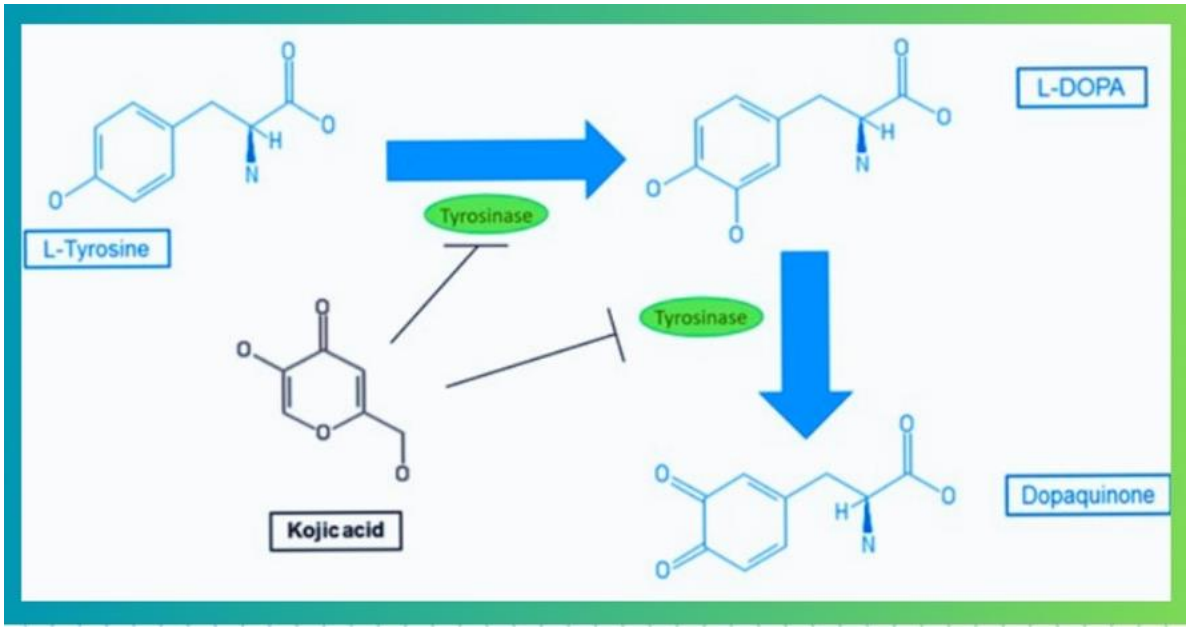

Figure 1.3: Overview of tyrosinase inhibition by kojic acid in melanin production. Chemical structures were drawn using PubChem Sketcher Version 2.4

KA chelating properties are crucial in the inhibition of tyrosinase activity. Copper (Cu^{2+}) and ferrous (Fe^{3+}) transition metals are chelated by KA. In cosmetics, KA chelates the copper ion (II) in tyrosinase, inhibiting the process and reducing the amount of melanin produced (Chen et al., 2013). KA scavenges free radicals, therefore, it can function as an anti-ageing agent and potentially diminish wrinkles (Saeedi et al., 2023).

Due to its hydrophilic nature, KA limits the compound's absorption in cosmetic applications. Permeability, bioavailability, and half-life of biological material are impacted by this characteristic (Saeedi et al., 2023). As a result of KA's inadequate skin penetration, solid lipid nanoparticles containing KA were created. Significant tyrosinase inhibition was observed in solid lipid nanoparticles compared to KA monotherapy (Khezri et al., 2020). Studies demonstrated that 3% KA was useful in lowering acne-related hyperpigmentation (Wawrzyk-Bochenek et al., 2023). Additionally, KA compounds with amides, triazole analogues, and phenol demonstrated reduced toxicity and more effective tyrosinase inhibition than KA alone (Noh et al., 2007, Noh et al., 2009, Ashooriha et al., 2019, Ashooriha et al., 2020). Other characteristics of KA may contribute to its anti-melanogenic action (Moon et al., 2001, Ma et al., 2011, Wawrzyk-Bochenek et al., 2023).

Hydroquinone is a potent tyrosinase inhibitor. Although hydroquinone has long-term negative effects such as nephrotoxicity and cancer, it was once the drug of choice for treating hyperpigmentation (Findlay et al., 1975, Kooyers and Westerhof, 2004). Hydroquinone is not utilized as a therapy for hyperpigmentation because of safety concerns. The FDA prohibited the use of hydroquinone in 2000

in favour of naturally occurring substitutes such as KA (Kwon et al., 2016, Chib et al., 2023). Due to its strong suppression of tyrosinase, which is frequently utilized as a positive control in studies, KA has grown in popularity (Chang, 2009).

2.2.4. Additional effects of kojic acid

KA studies have investigated several pharmaceutical and medical properties to determine potential applications. KA is commonly referred to as an anticancer, antioxidant, and anti-inflammatory agent (Brtko, 2022, Saeedi et al., 2019).

2.2.4.1. In vitro toxicity studies

2.2.4.1.1. Cancer

KA has been found to display anticancer properties (Nawarak et al., 2008, Lajis et al., 2012b, Chen et al., 2013, Suthiram et al., 2023). A summary of some of the effects of KA has been included in Figure 1.4.

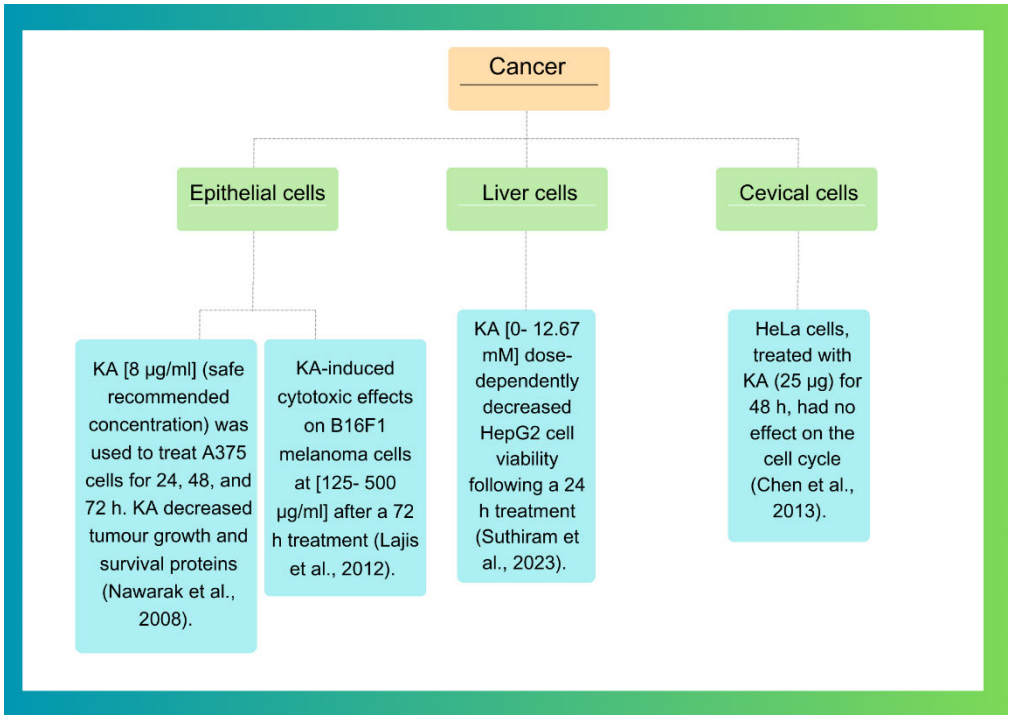

Figure 1.4: The effect of KA in *in vitro* cancer cells (Prepared by author)

2.2.4.1.2. Oxidative stress

Oxidative stress is a causative factor of many diseases. Compounds found to alleviate the burden of oxidative stress are beneficial to the treatment of disease. Some of the effects of KA are highlighted in Figure 1.5 (Niwa and Akamatsu, 1991, El-Metwally et al., 2020).

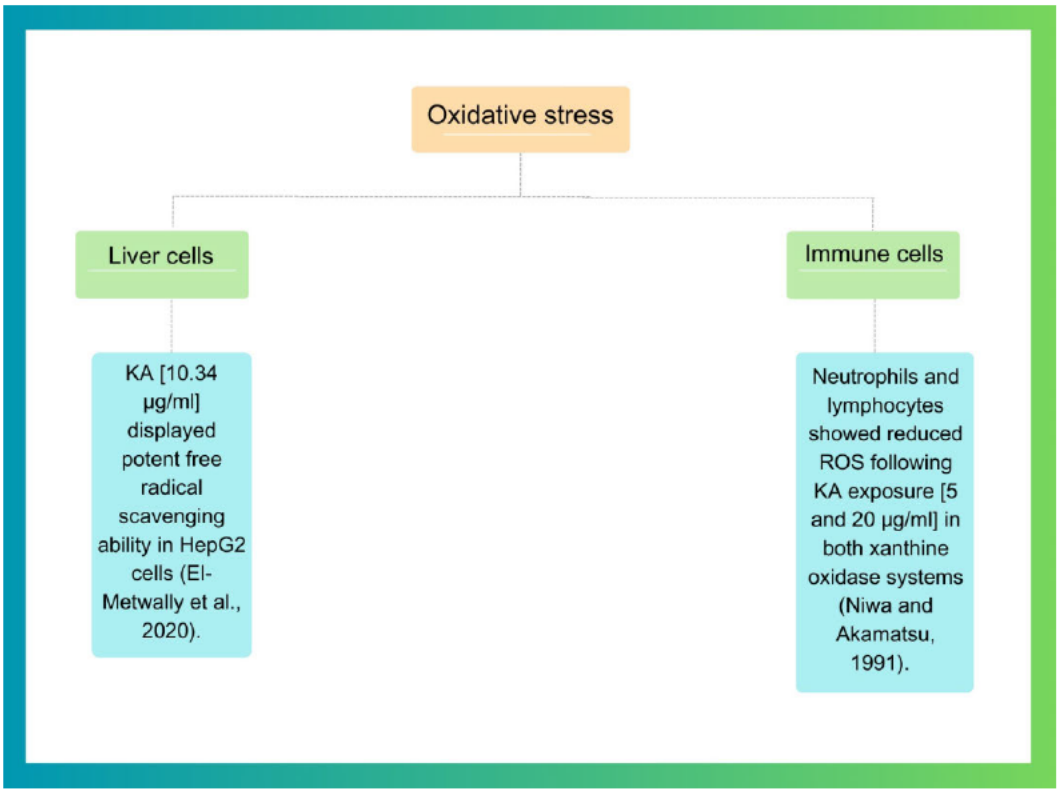

Figure 1.5: The effect of KA on oxidative stress markers (Prepared by author)

2.2.4.1.3. Inflammation

The activation of inflammatory pathways can work as a double-edged sword in terms of activating immune responses to protect the body or in contrast exasperating a disease state. KA has been found to exert anti-inflammatory effects in different cell lines (Table 1.2).

Table 1.2: The effect of KA on inflammatory markers in different cell lines

Cell lines	Biochemical effects	References
Epithelial cells	Human transfected-HaCaT and SCC-13 cells treated with 10 mM KA for 24 h showed inhibition of NFκB activation.	(Moon et al., 2001)
	KA [100 µM] was found to have senescence inhibition potential through NFκB and p21 pathways in human corneal epithelial cells (HCEC) following treatment for 1, 3, 5, and 7 days	(Wei et al., 2019)
	KA [0.2 mM] inhibited melanogenesis by stimulating interleukin-6 (IL-6) in keratinocyte and melanocyte co-cultures following a 3 day treatment.	(Choi et al., 2012)

Immune cells	KA [50 µg/ml] were not cytotoxic to monocytes and increased IL-6 production.	(Da Costa et al., 2018)
Liver cells	HepG2 cells treated with KA [8.02 mM] suppressed inflammatory marker, NFκB, following 24 h.	(Suthiram et al., 2023)

2.2.5. Clinical trials

For an average of 9.5 months, 107 chloasma patients were treated with KA creams (2.5% KA) twice a day to assess sensitivity. Two people experienced hypersensitivity that resulted in facial dermatitis. In 66 chloasma patients (Nakayama, 1982) and 31 healthy subjects when treated with the KA cream (1% KA) (Hira et al., 1985), no sensitivity was found. Studies conducted by Nakayama (1982) revealed that there was no sensitivity after using creams containing KA. In a similar investigation, patch testing on 220 female patients suspected of having cosmetic-associated dermatitis evaluated the prevalence of sensitization to KA. Eight patients out of the total used one KA skin product. Five responded to a product or items that contained 1% KA. The remaining three patients did not react to any KA-containing products. The 212 patients who had not previously used KA had no negative outcomes (Nakagawa et al., 1995). Another clinical study demonstrated the efficiency of 1% KA in treating melasma, freckles and age spots (Mishima et al., 1994, Ando et al., 2010). Limited research has been done to evaluate the impact of KA therapy alone.

In one study, melasma and hyperpigmentation were topically treated with a serum mixture of 3% tranexamic, 1% KA, and 5% niacinamide (Desai et al., 2019). Melasma and post-inflammatory hyperpigmentation responded well to the combination treatment. Forty women with melasma received a 12-week course of treatment consisting of 2% KA, 2% hydroquinone, and 10% glycolic acid using another combination therapy. Results showed that 24 women (or 60%) who underwent KA therapy had diminished melasma. These women's adverse reactions included redness, exfoliation, and skin sensitivity (Lim, 1999, Tetali et al., 2020).

When treating individuals with dyschromia, the combination of vitamin C and KA was found to be more effective than hydroquinone. Patients' dyschromia and skin tone improved while experiencing fewer side effects (Oresajo et al., 2008). A study by Draelos et al. (2010) evaluated the effectiveness of treating dyschromia patients with 4% hydroquinone in place of glycolic acid, Emblica plant extract, and KA. Eighty patients participated in the 12-week trial, and the results for both therapies were similar. As a result, the combination treatment provides a good alternative to hydroquinone and was just as effective (Tetali et al., 2020, Draelos et al., 2010). Hyperpigmentation was treated with KA combination therapy in an additional clinical trial involving 39 patients. One-half of the patients' faces received treatment with 5% glycolic acid and 2% KA, whereas the other half received treatment with 5% glycolic acid and

hydroquinone. According to the findings, 28% of the participants observed improvement on the KA side and 21% found improvement on the hydroquinone side (Gupta et al., 2006).

2.3. Mitochondrion

2.3.1. Mitochondrial function

Mitochondria are double membrane organelles that are present in eukaryotic organisms. The organelle can be divided into three sections, the outer membrane, the inner membrane, and the intermembrane space known as the matrix. The mitochondria are a hub for signalling pathways and play a crucial role in cellular respiration. Approximately 98% of oxygen inhaled is consumed by the mitochondria. Mitochondria supply energy, in the form of ATP, required for many processes such as muscle movement, maintenance of ionic gradients, and secretion of hormones and neurotransmitters. The production of free radicals by the mitochondria serves as a secondary messenger that initiates mechanisms to alleviate cellular stress. One of the major roles of the mitochondria is the generation of transmembrane potential across the inner membrane to form an energy gradient (Duchen, 2004, Sies et al., 2017).

Several substrates (pyruvate, amino acids, products formed from β-oxidation of fatty acids) enter the mitochondria and are used in the tricarboxylic acid cycle and maintain reducing equivalent ratios ($NADH/NAD^+$, $FADH_2/FAD$). These reducing equivalents transfer hydrogen to oxygen and generate proton transfer across the mitochondrial inner membrane resulting in a proton gradient. This allows for ATP generation by driving ATP synthase conversion of adenine diphosphate (ADP) to ATP. ATP is then transferred to the cytosol via the adenine nucleotide translocase (Duchen, 2004).

2.3.2. Oxidative stress

The mitochondria are responsible for producing the highest amount of ROS in mammalian cells. The electron transport chain produces ROS through Complexes I and III. Mitochondrial ROS is only produced by these complexes (Chouchani et al., 2014). Superoxide can be converted into H_2O_2 with the help of superoxide dismutase (SOD) through a process called dismutation (Chen et al., 2021). Mitochondrial dysfunction resulting from excess ROS production can impair an organism's ability to sustain life, leading to disorders and diseases. Mitochondrial ROS serves as a signalling molecule, but high levels can damage DNA, protein, and lipids (Schieber and Chandel, 2014, Mailloux, 2020). Efficient cell functioning is required to regulate ROS production.

Oxidative stress is defined as the imbalance of prooxidants and oxidants (Sies et al., 2017). Nitrous oxide or O_2 superoxide generation in the mitochondria can both cause free radical production, which in turn can change the assembly of the electron transport chain or cause hyperproliferation (Hayes et al., 2020). Increased ROS can inactivate tumour suppressor transcription factor (p53), which lowers SOD_2

and glutathione peroxidase (GPx) expression (Hayes et al., 2020). The increased levels of ROS encourage the proliferation of cells by altering ERK, PI_3K/Akt and NFκB signalling (Moloney and Cotter, 2018).

2.3.3. Master regulators of redox homeostasis

Biological systems possess redox switches enabling the activation or deactivation of redox signalling and redox sensing. Multiple H_2O_2 transcription factors sense changes in redox systems and modulate biological pathways. These transcription factors, referred to as master regulators, detoxify oxidized by-products, repair, and control cellular function. Cellular homeostasis is stringently maintained by controlling cell proliferation, and apoptosis (Marinho et al., 2014, Espinosa-Diez et al., 2015). These master regulators include the Nrf-2 and NFκB signalling pathways.

2.3.4. Antioxidant responses- Nrf-2 activation

Nrf-2 forms part of a family of transcription factors responsible for the initiation of antioxidant responses via detoxification enzymes (Itoh et al., 1997, Sies et al., 2017). Under normal conditions, KEAP-1 regulates antioxidant responses by ubiquitinating and degrading Nrf-2. KEAP-1 contains sulfhydryl groups that detect oxidants (Dinkova-Kostova et al., 2002). Phosphorylation of KEAP-1 at the tyrosine 141 residue stabilises the protein. Under oxidative conditions, the cysteine residues of KEAP-1 are modified, altering the protein's ability to ubiquitinate Nrf-2. KEAP-1 is dephosphorylated in the presence of H_2O_2 leading to degradation of KEAP-1 and activation of Nrf-2 (Jain et al., 2008, Espinosa-Diez et al., 2015).

When Nrf-2 accumulates in the nucleus, it leads to the transcription of genes responsible for producing antioxidants (Sporn and Liby, 2012, Sies et al., 2017). Studies evaluating KEAP-1 and Nrf-2 activity in KEAP-1 knockout mice found that mice died shortly after birth. The study further found that double knockout of Nrf-2 and KEAP-1 reversed the altered phenotype. This result shows that Nrf-2 responses, when either elevated or depleted in excess, can be detrimental (Wakabayashi et al., 2003, Espinosa-Diez et al., 2015).

2.4. Mitochondrial homeostasis

Mitochondrial homeostasis is the balance between mitochondrial biogenesis and mitophagy. These two processes must be highly regulated to ensure efficient cell function. There are several proteins involved in the upkeep and maintenance of mitochondrial machinery (Popov, 2020). Another aspect of antioxidant defence is the removal of oxidised proteins. Free radicals interact with protein side chains resulting in altered function. Damaged proteins need to be removed before they aggregate and form complexes by cross-linking (Ngo and Davies, 2007). For our research, we focussed on LON protease and Sirtuin 3 functionality.

2.4.1. LON proteases

LON protease is an ATP-dependent protein that is found in the mitochondria and peroxisomes of eukaryotes (Lee and Suzuki, 2008). When antioxidant responses are insufficient or bypassed, LON protease plays a key role in the removal of oxidised proteins in the mitochondria (Bota and Davies, 2002, Ngo and Davies, 2007).

Hydrophobic amino acids interact with the N-terminus of LON. Subsequently, ATP hydrolysis ensues allowing for the translocation of the protein substrate to the proteolytic chamber for protein cleavage. The proteasome is the most prominent proteolytic system in mammals (Ondrovicová et al., 2005). LON binds to the promotor region of mitochondrial DNA. The binding of LON to DNA is prevented when LON is bound to ATP. In the presence of oxidative conditions, DNA binding is weakened and oxidised proteins are the preferred substrate. The LON complex changes conformation and uses ATP to degrade the oxidised protein or unfolded peptides. In this way, LON can switch functions from nucleic acid binding to oxidised protein binding (Ngo and Davies, 2007). LON protease is in the mitochondrial matrix where there is a high free radical concentration. The mitochondria DNA and protein are particularly vulnerable to damage due to the proximity to the electron transport chain. LON acts as a sensor that regulates mitochondrial homeostasis (Ngo and Davies, 2007). LON protease is a stress response protein that is regulated by Sirtuin 3 (Gibellini et al., 2014).

2.4.2. Sirtuins

The Sirtuin family in mammals have seven members (Sirtuin 1-7) (Figure 1.6). They have a core domain consisting of 275 amino acids with an N-terminal and C-terminal of varying lengths (Michan and Sinclair, 2007). Sirtuins function in different sub-cellular locations such as the nucleus (Sirtuin 1, 6 and 7), cytoplasm (Sirtuin 2), and mitochondria (Sirtuin 3, 4, and 5). Sirtuins require NAD^+ to effectively function. Sirtuin 1 performs potent deacetylase activity and some cytoplasmic functions. Sirtuins 2 and 3 deacetylate mitochondrial proteins and perform mono-ADP-ribosyl transferase activity (Carrico et al., 2018). Sirtuin 5 have weak deacetylase activity while Sirtuin 4 and 6 are mono-ADP-ribosyl transferases (Michan and Sinclair, 2007).

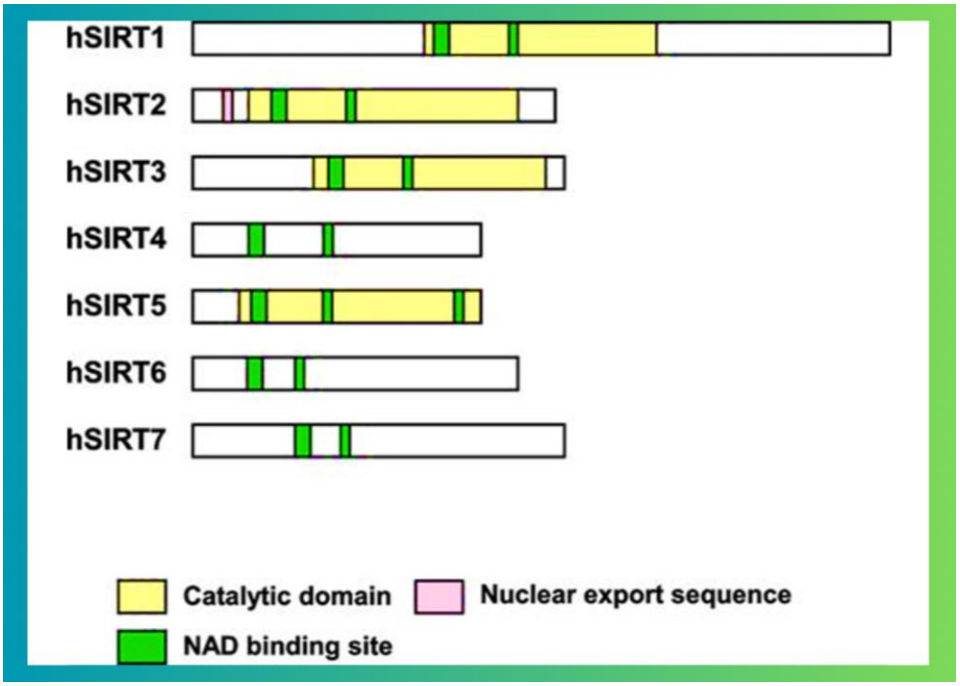

Figure 1.6: Structure of Sirtuin proteins found in mammals (Yamamoto et al., 2007)

2.4.2.1. Sirtuin 3

Sirtuin 3 performs several roles such as decreasing mitochondrial membrane potential, reducing free radical production, increasing cellular respiration and initiating global acetylation of mitochondrial proteins (Hebert et al., 2013). Moreover, Sirtuin 3 has an important role as a deacetylase in controlling several activities, including mitochondrial metabolism, oxidative stress and inflammation suppression, apoptosis and autophagy regulation, and mitochondrial homeostasis maintenance (Torrens-Mas et al., 2019, Zhang et al., 2020, Schwer and Verdin, 2008b, Anderson et al., 2014). Sirtuin 3 exhibits both anti- and pro-tumour properties. The p53 protein can be deacetylated by Sirtuin 3. Deacetylation of p53 can result in its breakdown, which allows cancer cells to proliferate (Xiong et al., 2018). On the other hand, Sirtuin 3 stimulates mitochondrial activity by initiating the deacetylation of pyruvate dehydrogenase. This lowers the rate of cancer cell proliferation (Yapryntseva et al., 2022). Sirtuin 3 also upregulates the gene expression of uncoupling protein 1 (UCP1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), cytochrome c oxidase subunits II and IV, and ATP synthase (Michan and Sinclair, 2007).

Regulation of the Sirtuin 3 protein is vital in ensuring efficient mitochondrial metabolism and reduced oxidative stress. Nrf-2 transcriptionally activates the expression of Sirtuin 3 in high oxidative conditions (Satterstrom et al., 2015). Sirtuin 3 is known to increase oxidative phosphorylation and inhibit the Warburg effect (Finley et al., 2011). A pH-dependent interaction occurs between Sirtuin 3 and ATP synthase in the mitochondrial inner membrane. If the pH changes and mitochondrial depolarisation occurs, Sirtuin 3 will not interact with ATP synthase, due to protonation of ATP50 subunits, but will

deacetylate mitochondrial proteins (Yang et al., 2016). The mitochondrial proteins will assist in restoring the mitochondria to normal condition. In this way, Sirtuin 3 aids in maintaining mitochondrial homeostasis (Wang and Lin, 2021). Additionally, Sirtuin 3 deacetylates and activates mitochondrial AceCS2. This enzyme is responsible for the conversion of acetate to acetyl CoA in the tricarboxylic acid cycle. NAD^+ serves as a substrate for Sirtuin 3 deacetylation reactions (Carrico et al., 2018). NAD^+ catalyses the transfer of electrons in oxidation-reduction reactions involved in glycolysis and the tricarboxylic acid cycle. The NAD^+/NADH ratio dictates the efficiency of energy production (Stein and Imai, 2012). Therefore, Sirtuin 3 plays a key role in ATP generation (Yamamoto et al., 2007).

2.5. Cell signalling pathways

2.5.1. Protein kinase signalling

Signalling pathways such as the MAPK (ERK 1/2 and p38), Akt and GSK3β phosphorylate MITF which influence melanin production. Protein kinases form a group of enzymes responsible for phosphorylating tyrosine, threonine/serine residues found in target proteins. Phosphorylation of these residues leads to altered function. Serine/threonine protein kinases containing cysteine are susceptible to modifications by ROS (Espinosa-Diez et al., 2015). This suggests that KA, a known tyrosinase inhibitor, could potentially affect these protein kinases (Perdomo et al., 2020).

2.5.1.1. MAPK signalling

The mitogen-activated protein kinases (MAPKs) are serine/threonine kinases that catalyse gene expression, cell proliferation, and apoptosis (Espinosa-Diez et al., 2015). MAPK signalling is activated by a phosphorylation cascade involving threonine and tyrosine residues found within a tripeptide motif with dual specificity.

MAPK can be activated by ROS through the inactivation and degradation of MAPK phosphatases. Free radicals (H_2O_2) oxidise catalytic cysteine residues which activate the enzyme resulting in the activation of MAPK signalling. Contrary to the findings, H_2O_2 can enhance the expression of MAPK phosphatase and inactivate MAPK proteins, JNK and p38. The dichotomy of studies highlights the role of ROS in acting as an inducer and suppressor of MAPK (Marshall, 1994, Zhou et al., 2006). The concentrations of ROS may play an integral part in the contrasting results, suggesting that cysteine residue's redox sensitivity is key to MAPK phosphatase activity and subsequently MAPK signalling.

MAPK signalling can activate Nrf-2 expression which in turn initiates the transcription of antioxidant response element-mediated genes. Examples of these MAPK-dependent pathways are:

1) The upregulation of peroxiredoxins (Prx) I antioxidant responses due to the upregulating of p38 signalling (Kim et al., 2013b).

2) The regulation of NFκB through the phosphorylation of IκB-alpha and p65 NFκB resulting in enhanced inducible nitric oxide synthase (iNOS) and activator protein-1 (AP-1) gene expression (Dhar et al., 2002).

The regulation and activation of MAPK proteins may be cell-specific and ROS concentration-dependent. In smooth muscle, intracellular ROS activates p38 and ERK 5 whereas ERK 1/2 activation is redox sensitive (Espinosa-Diez et al., 2015, Catarzi et al., 2011).

2.5.1.1.1. p38

The p38 protein is a stress-activated protein kinase that is activated by environmental and genotoxic factors. It is encoded by four genes, namely p38α (MAPK14), p38β (MAPK11), p38γ (MAPK12) and p38ζ (MAPK13) (Cuenda and Rousseau, 2007, Cuenda and Sanz-Ezquerro, 2017). The p38α and p38β isoforms are 75% similar and are expressed in all tissues whereas p38γ and p38ζ are 70% similar and expressed in specific tissues. The dual activation at threonine and tyrosine residues alters the folding of p38. MAP2Ks (MKK3 and MKK6) are responsible for the phosphorylation of p38 (Doza et al., 1995, Dérijard et al., 1995). These kinases are activated by MAP3Ks such as apoptosis signal-regulating kinase 1 (ASK1), dual leucine-zipper-bearing kinase-1 (DLK1), transforming growth factor β-activated kinase 1 (TAK1), MEKK (MAPK/ERK kinase kinase) and several other proteins (Martínez-Limón et al., 2020).

2.5.1.1.2. Extracellular signal-regulated kinase 1/2 (ERK 1/2)

There are five members of the ERK family (ERK1-5), with ERK1 and ERK2 being the most studied. These two members of the family share a 90% similarity in homology (Boulton and Cobb, 1991, Kong et al., 2019).

Activation of ERK1/2 occurs through a three-step reaction involving MAPKKK (Raf), MAPKK (ERK1/2 kinase) and MAPK (ERK1/2) (Figure 1.7). The ERK1/2 enzyme is located in the cytoplasm. Stimuli initiates the phosphorylation of ERK1/2 at serine/threonine residues resulting in the regulation of cell growth, proliferation, and differentiation. These stimuli include inflammation, growth factors, neurotransmitters, ischemia, and hypoxia. The classical ERK1/2 pathway is activated via receptors that will activate tyrosine kinases. These kinases will in turn activate Ras. Ras that is bound to GTP will activate Raf that will translocate from the cytoplasm to the membrane and activate MEK at its serine residue. Lastly, MEK will activate ERK1/2, which will activate target proteins and enter the nucleus to activate the transcription of specific genes. ERK1/2 plays a role in apoptosis, autophagy, proliferation, cell cycle, cell growth, cell differentiation, tumorigenesis, oxidative stress and inflammation (Kong et

al., 2019, Asati et al., 2016, Overmeyer and Maltese, 2011, Zaleśna et al., 2016, Coleman et al., 2004, Kim et al., 2014, Sivaprasad and Basu, 2008).

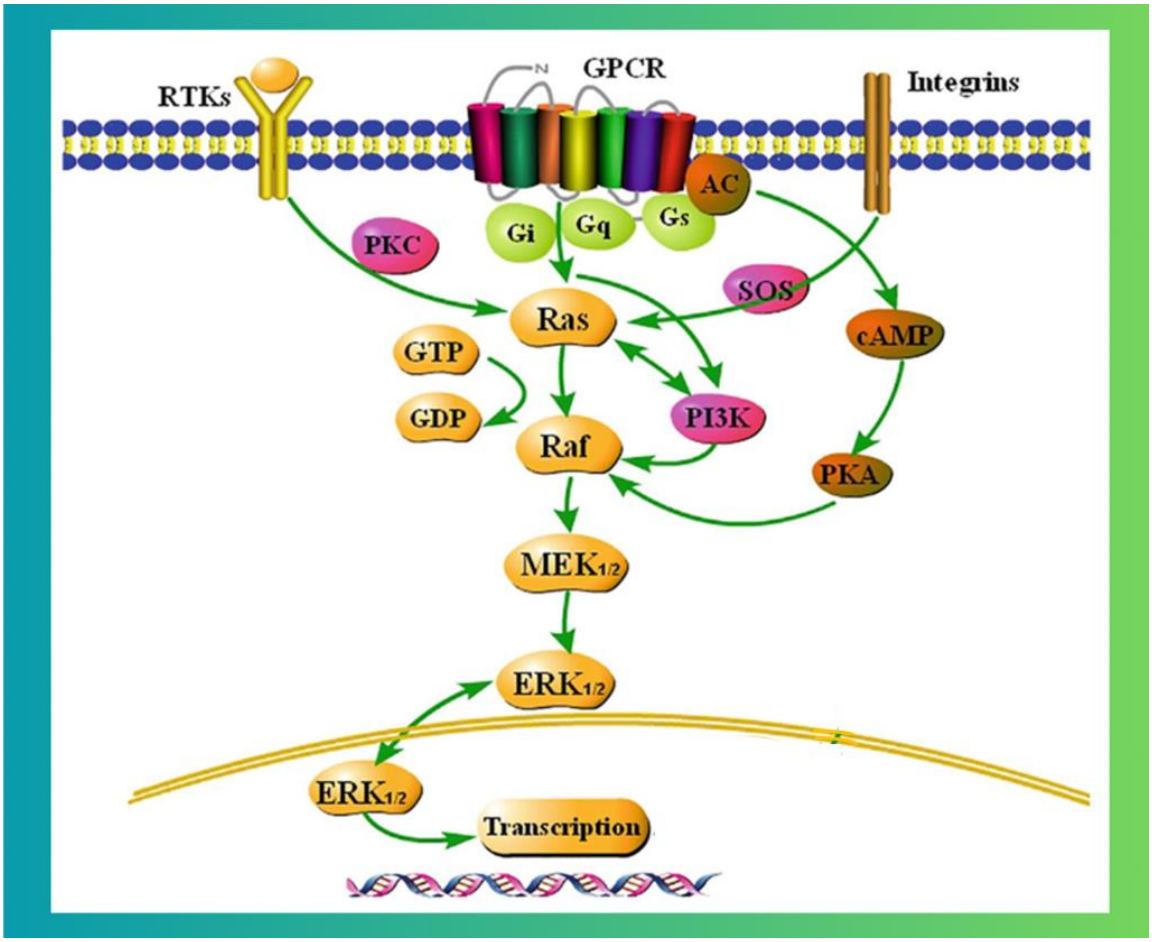

Figure 1.7: The classical three-step cascade of reactions employed to activate ERK1/2 (Kong et al., 2019)

2.5.1.2. PI_3K/Akt signalling pathway

Akt is a serine/threonine kinase that catalyses growth factors involved in cell survival. Activated Akt leads to the suppression of apoptotic machinery. There are three Akt genes (*Akt1*, *Akt2*, and *Akt3*) in the human genome, with *Akt1* being the primary gene for the modulation of apoptosis. Akt is activated by growth factors and cytokines at the threonine and serine hydrophobic motifs. Once activated, Akt follows a step process of inducing survival factors, translocating to the plasma membrane and finally phosphorylating downstream processes (Datta et al., 1999).

Translocation of Akt from the cytoplasm to the cell membrane is important for Akt phosphorylation. This process relies on the phospholipids generated by PI_3K, and PIP_3 binding of Akt. When PIP_3 directly binds to the amino terminus of Akt, a conformational change occurs, which allows PDK-1 to

phosphorylate the Thr308 residue. PIP_3 is inhibited by PTEN which suppresses PI_3K signalling (Revathidevi and Munirajan, 2019, Alessi et al., 1998).

The downstream effects of Akt include the phosphorylation and activation of IKKα at its regulatory site, Thr23, resulting in the phosphorylation and ubiquitination of IκB. Therefore, NFκB is free to translocate to the nucleus to activate gene transcription of inflammation, cell cycle and apoptosis-related genes (Nidai Ozes et al., 1999, Revathidevi and Munirajan, 2019). Furthermore, Akt phosphorylates proteins involved in glucose metabolism. The most well-known substrate of Akt is GSK-3. GSK-3 is an enzyme that plays a role in glycogen synthesis in response to insulin, by regulating glycogen synthase which in turn regulates cellular metabolism. Akt shares an inversely proportional relationship with GSK-3 and can inactivate its phosphorylating ability (Pap and Cooper, 1998, Revathidevi and Munirajan, 2019, Welsh et al., 1996).

2.5.2. Role of cell signalling in Nrf-2 regulation

In facilitating an antioxidant response, Nrf-2 binds to a β-transducing repeat-containing protein (β-TrCP) at the Neh6 domain. This binding can be phosphorylated by GSK-3β leading to the ubiquitination and degradation of Nrf-2, facilitated by Cullin 1. This is a KEAP-1-independent means of Nrf-2 degradation (Figure 1.8). GSK-3β is activated by H_2O_2 and inhibited by phosphorylation facilitated by cell signalling proteins such as Akt, ERK and p38. There is also cross-talk between Nrf-2 and other pathways such as NFκB and p53 (Espinosa-Diez et al., 2015, Chowdhry et al., 2013, McMahon et al., 2004).

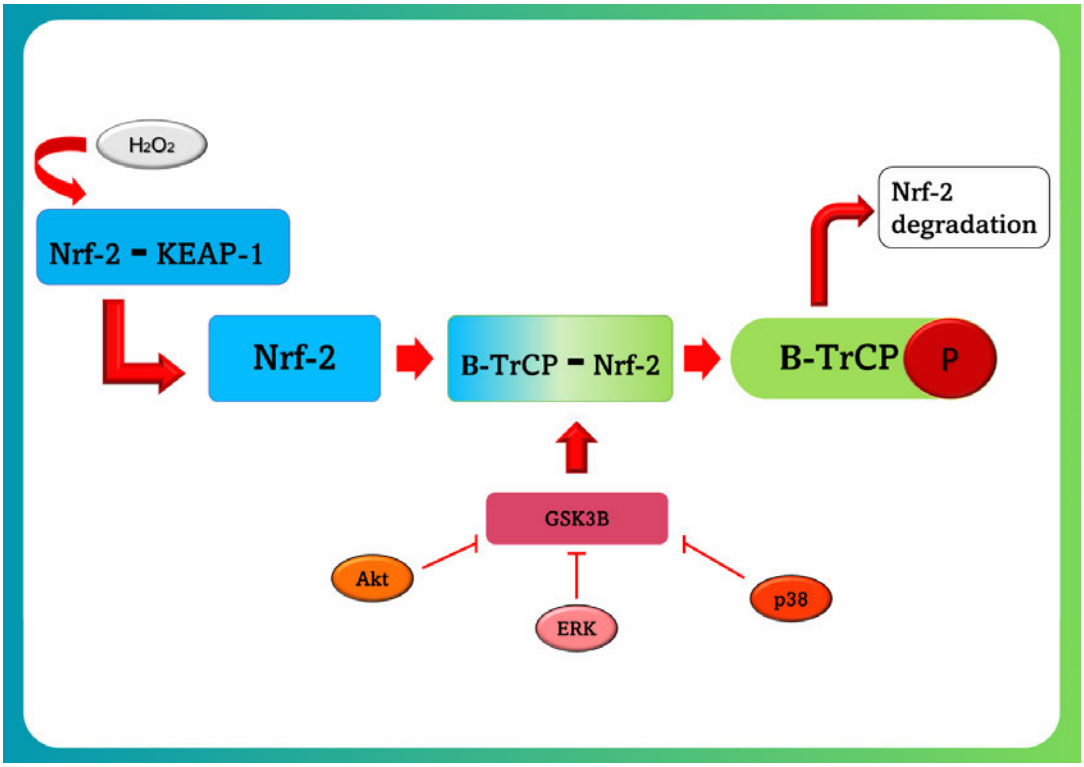

Figure 1.8: Schematic illustration of the role of ROS and signalling pathways in the KEAP-1 independent regulation of Nrf-2 (Prepared by author)

2.6. Inflammation

2.6.1. NFκB inflammatory pathway

The activation of NFκB is a common inflammatory pathway employed in skin disease. The NFκB family are transcription factors that induce immune, inflammation and anti-apoptotic responses (Karin, 2006). The NFκB family is made up of five proteins: (1) NFκB1 (p50), (2) NFκB2 (p52), (3) Rel A (p65), (4) Rel B, and (5) c-Rel (Sun et al., 2013). NFκB is activated by canonical and non-canonical pathways that are both important for immune and inflammatory responses.

The canonical pathway is activated by stimuli and ligands (cytokine receptors, TNF receptors, pattern recognition receptors (PRR), T-cell and B-cell receptors) (Zhang and Sun, 2015). NFκB is activated by the phosphorylation of the IKK complex and the ubiquitin-dependent degradation of IκBα in the proteasome. The IKK complex is made up of three subunits namely: two catalytic subunits, that are IKKα and IKKβ and a regulatory subunit, IKKγ, which is also termed NFκB essential modulator (NEMO). NFκB members, p50/Rel A and p50/c-Rel dimers leave the nucleus (Sun and Ley, 2008, Sun et al., 2013).

The non-canonical pathway relies on NFκB2 (p100) rather than IκB degradation. The NFκB-inducing kinase activates IKKα and works together to phosphorylate NFκB, precursor protein, p100. This leads

to p100 ubiquitination and processing. The term processing encompasses the degradation of p100 at the C-terminal and the activation of p52/Rel B (Sun, 2012, Sun et al., 2013).

The functions of the two pathways differ; the canonical pathway is involved in immune responses and the non-canonical pathway co-operates with the canonical pathway in regulating functions in the adaptive immune system (Liu et al., 2017).

2.6.2. Inflammasome

Pattern recognition receptors in tissue cells, such as innate immune cells, detect pathogens and damage and activate pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (Medzhitov and Janeway, 1997, Matzinger, 1994, Groslambert and Py, 2018). Inflammatory cytokines, IL-6, tumour necrosis factor (TNF-α) and interferon-β (IFN-β) are transcriptionally regulated and secreted through the endoplasmic reticulum (ER)/golgi pathway. IL-1β and IL-18 are cytosolic precursors that are controlled by caspase 1, 4 and 5 in humans. NLRP3 acts as a sensor for cellular homeostasis and assembles a multi-protein inflammasome which activates caspases leading to the direct cleavage and maturation of pro-cytokines such as pro-IL-1β (Figure 1.9) (Groslambert and Py, 2018).

2.6.2.1. Control of NLRP3 assembly

2.6.2.1.1 Signal 1- Priming

Many cytokines and receptors control the assembly of the NLRP3 inflammasome. These receptors that prime NLRP3 for activation include Toll-like receptors (TLR), receptors for advanced glycation end products (RAGEs), and nucleotide-binding oligomerization domain 2 (NOD2) (Alexiou et al., 2010, Xiang et al., 2011, Fritz et al., 2006, Meylan et al., 2006, Mitchell et al., 2007). Priming of NLRP3 can occur via the immune system through anaphylatoxin (C3a and C5a) receptors, cytokines (IL-1 and TNF-α) and ROS. Transcriptional priming is dependent on factors such as myeloid differentiation primary response 88 (MyD88), TIR domain-containing adaptor-inducing interferon-β (TRIF), caspase 8, Fas-associated protein with death domain (FADD), NFκB and ROS (Gurung et al., 2014, Zhang et al., 2016, Bauernfeind et al., 2011). Upon the activation of TLR, caspase 1 and pro-IL-1β are highly expressed (Bauernfeind et al., 2009). Non-transcriptional priming relies on post-translational modifications (Groslambert and Py, 2018). In the priming stage, signalling pathways following the activation of receptors can follow some of the following fates.

1) NLRP3 change due to post-translational modification leading to activation of the inflammasome.
2) Late transcriptional activation of NLRP3, pro-IL-1β and several other regulators.
3) Prolonged activation leads to a targeted suppression of inflammasome activators.

2.6.2.1.2. Signal 2- Activation

Activation of the inflammasome is mediated under an array of conditions. The activation signals are classified according to the type of stress they initiate. The classes include agents that change the plasma membrane permeability e.g., pore-forming bacterial toxins (Cookson and Brennan, 2001), crystals e.g., asbestos (Hornung et al., 2008, Zhong et al., 2013), and particulates released from cells. The first class of activators performs their function by permeabilising the plasma membrane and opening ionic pores (Aglietti et al., 2016, Rühl and Broz, 2015). Crystals undergo phagocytosis but not degradation which causes the accumulation of crystals resulting in phagolysosome lysis, releasing of Ca^{2+} and cathepsin in the cytoplasm (Triantafilou et al., 2013, Murakami et al., 2012). Lastly, particulates expelled by pyroptotic cells (inflammasome specks) are released and inflammation is amplified (Baroja-Mazo et al., 2014, Franklin et al., 2014). Several other activators have been discovered to activate the inflammasome such as ultraviolet radiation.

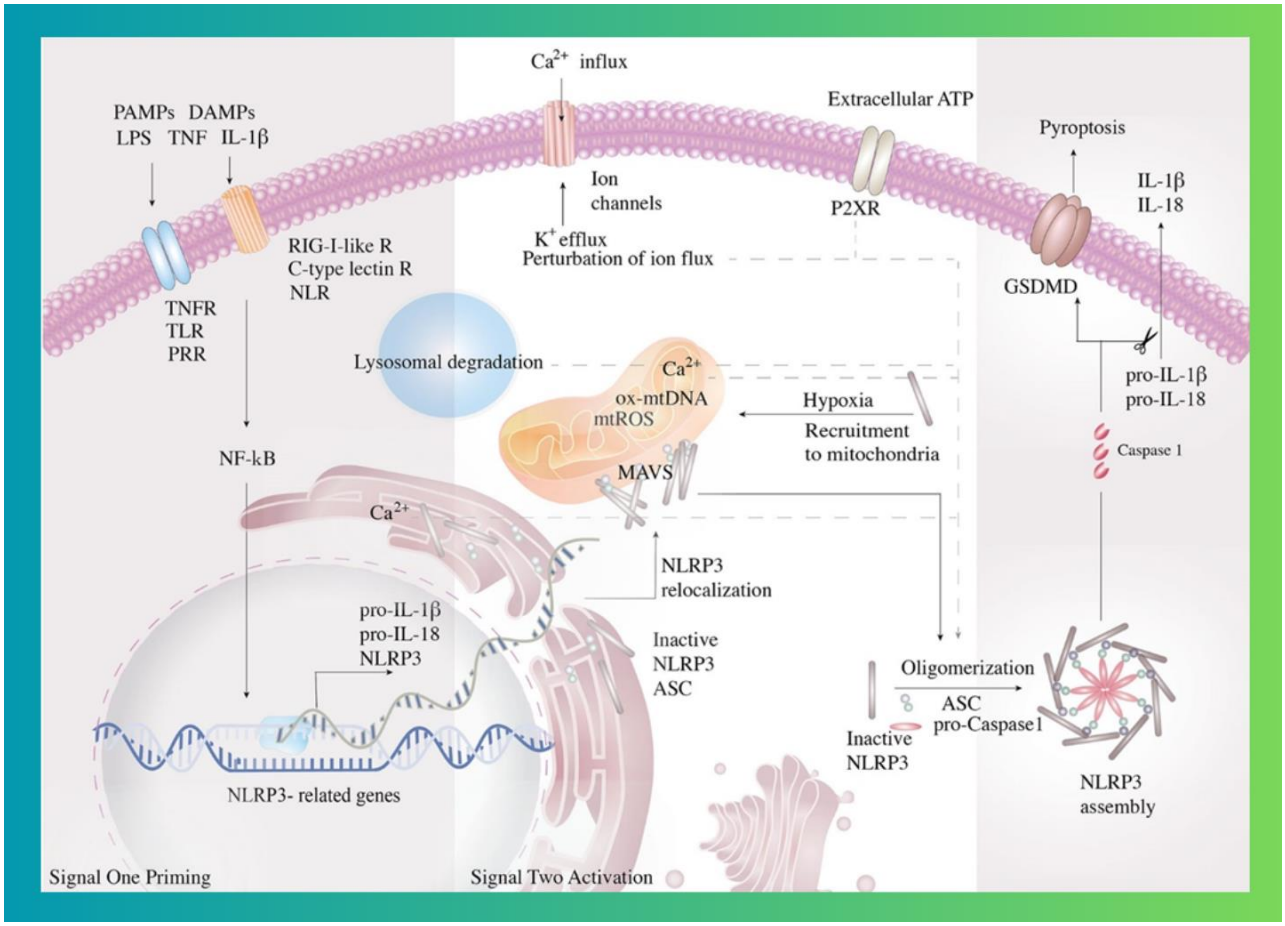

Figure 1.9: The process of priming and activation of the NLRP3 inflammasome (Lin et al., 2021)

2.6.3. Inflammation and skin

NFκB signalling transcribes proinflammatory molecules that can lead to inflammatory skin diseases. Many proinflammatory cytokines have NFκB binding sites. In the case of dermatitis, NFκB is

continuously activated due to deficient IκB levels (Sur et al., 2008). In elevated ROS conditions, the activation of MAPK proteins (ERK AND JNK) through MEK is heightened to increase the recruitment of c-Fos and c-Jun resulting in the activation of AP-1. This supplies the cell with antioxidant genes required for alleviating oxidative stress. Another important MAPK protein (p38) is involved in the activation of NFκB and regulates genes involved in antioxidant responses (Espinosa-Diez et al., 2015).

C-reactive proteins (CRP) are a cytokine that interacts with the PI_3K/Akt signalling pathway. It binds to cytokine receptors and activates PI_3K which thereafter mediates protein kinase signalling. The activation of Akt initiates the phosphorylation of ERK, metabolism proteins and processes that influence cell proliferation, metabolism, and cell death (She et al., 2018, Revathidevi and Munirajan, 2019). Similarly, the NLRP3 inflammasome plays a role in the release of cytokines, key causative agents in skin diseases such as vitiligo, alopecia, and psoriasis (Fetter et al., 2023).

2.7. References

AGLIETTI, R. A., ESTEVEZ, A., GUPTA, A., RAMIREZ, M. G., LIU, P. S., KAYAGAKI, N., CIFERRI, C., DIXIT, V. M. & DUEBER, E. C. 2016. GsdmD p30 elicited by caspase-11 during pyroptosis forms pores in membranes. *Proceedings of the National Academy of Sciences,* 113**,** 7858-7863.

ALESSI, D. R., JAMES, S. R., DOWNES, C. P., HOLMES, A. B., GAFFNEY, P. R., REESE, C. B. & COHEN, P. 1997. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Bα. *Current biology,* 7**,** 261-269.

ALESSI, D. R., KOZLOWSKI, M. T., WENG, Q. P., MORRICE, N. & AVRUCH, J. 1998. 3-Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase in vivo and in vitro. *Current Biology,* 8**,** 69-81. DOI: 10.1016/s0960-9822(98)70037-5.

ALEXIOU, P., CHATZOPOULOU, M., PEGKLIDOU, K. & DEMOPOULOS, V. 2010. RAGE: a multi-ligand receptor unveiling novel insights in health and disease. *Current medicinal chemistry,* 17**,** 2232-2252.

AMSDEN, B. & GOOSEN, M. 1995. Transdermal delivery of peptide and protein drugs: an overview. *AIChE journal,* 41**,** 1972-1997.

ANDERSON, K. A., GREEN, M. F., HUYNH, F. K., WAGNER, G. R. & HIRSCHEY, M. D. 2014. SnapShot: Mammalian Sirtuins. *Cell,* 159**,** 956-956.e1. DOI: 10.1016/j.cell.2014.10.045.

ANDO, H., MATSUI, M. S. & ICHIHASHI, M. 2010. Quasi-Drugs Developed in Japan for the Prevention or Treatment of Hyperpigmentary Disorders. *International Journal of Molecular Sciences,* 11**,** 2566-2575.

ASATI, V., MAHAPATRA, D. K. & BHARTI, S. K. 2016. PI3K/Akt/mTOR and Ras/Raf/MEK/ERK signaling pathways inhibitors as anticancer agents: Structural and pharmacological perspectives. *European journal of medicinal chemistry,* 109**,** 314-341.

ASHOORIHA, M., KHOSHNEVISZADEH, M., KHOSHNEVISZADEH, M., MORADI, S. E., RAFIEI, A., KARDAN, M. & EMAMI, S. 2019. 1,2,3-Triazole-based kojic acid analogs as potent tyrosinase inhibitors: Design, synthesis and biological evaluation. *Bioorganic Chemistry,* 82**,** 414-422. DOI: 10.1016/j.bioorg.2018.10.069.

ASHOORIHA, M., KHOSHNEVISZADEH, M., KHOSHNEVISZADEH, M., RAFIEI, A., KARDAN, M., YAZDIAN-ROBATI, R. & EMAMI, S. 2020. Kojic acid-natural product conjugates as mushroom tyrosinase inhibitors. *European Journal of Medicinal Chemistry,* 201**,** 112480. DOI: 10.1016/j.ejmech.2020.112480.

BABER, M. A., CRIST, C. M., DEVOLVE, N. L. & PATRONE, J. D. 2023. Tyrosinase Inhibitors: A Perspective. *Molecules,* 28**,** 5762.

BAROJA-MAZO, A., MARTÍN-SÁNCHEZ, F., GOMEZ, A. I., MARTÍNEZ, C. M., AMORES-INIESTA, J., COMPAN, V., BARBERÀ-CREMADES, M., YAGÜE, J., RUIZ-ORTIZ, E. & ANTÓN, J. 2014. The NLRP3 inflammasome is released as a particulate danger signal that amplifies the inflammatory response. *Nature immunology,* 15**,** 738-748.

BARRANDON, Y., GRASSET, N., ZAFFALON, A., GOROSTIDI, F., CLAUDINOT, S., DROZ-GEORGET, S. L., NANBA, D. & ROCHAT, A. 2012. Capturing epidermal stemness for regenerative medicine. *Semin Cell Dev Biol,* 23**,** 937-44. DOI: 10.1016/j.semcdb.2012.09.011.

BASRA, M. K. A. & SHAHRUKH, M. 2009. Burden of skin diseases. *Expert Review of Pharmacoeconomics & Outcomes Research,* 9**,** 271-283. DOI: 10.1586/erp.09.23.

BAUERNFEIND, F., BARTOK, E., RIEGER, A., FRANCHI, L., NÚÑEZ, G. & HORNUNG, V. 2011. Cutting edge: reactive oxygen species inhibitors block priming, but not activation, of the NLRP3 inflammasome. *The Journal of Immunology,* 187**,** 613-617.

BAUERNFEIND, F. G., HORVATH, G., STUTZ, A., ALNEMRI, E. S., MACDONALD, K., SPEERT, D., FERNANDES-ALNEMRI, T., WU, J., MONKS, B. G. & FITZGERALD, K. A. 2009. Cutting edge: NF-κB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *The Journal of Immunology,* 183**,** 787-791.

BEARD, R. L. & WALTON, G. S. 1969. Kojic acid as an insecticidal mycotoxin. *Journal of Invertebrate Pathology,* 14**,** 53-59. DOI: https://doi.org/10.1016/0022-2011(69)90010-X.

BEELIK, A. 1956. Kojic acid. *Advances in Carbohydrate Chemistry and Biochemistry,* 48**,** 145-183.

BENTLEY, R. 2006. From miso, saké and shoyu to cosmetics: a century of science for kojic acid. *Natural Products Reports,* 23**,** 1046-1062. DOI: 10.1039/b603758p.

BLUME-PEYTAVI, U., KOTTNER, J., STERRY, W., HODIN, M. W., GRIFFITHS, T. W., WATSON, R. E. B., HAY, R. J. & GRIFFITHS, C. E. M. 2016. Age-Associated Skin

Conditions and Diseases: Current Perspectives and Future Options. *The Gerontologist,* 56**,** S230-S242. DOI: 10.1093/geront/gnw003.

BOTA, D. A. & DAVIES, K. J. 2002. Lon protease preferentially degrades oxidized mitochondrial aconitase by an ATP-stimulated mechanism. *Nature Cell Biology,* 4**,** 674-80. DOI: 10.1038/ncb836.

BOULTON, T. G. & COBB, M. H. 1991. Identification of multiple extracellular signal-regulated kinases (ERKs) with antipeptide antibodies. *Cell Regulation,* 2**,** 357-371. DOI: 10.1091/mbc.2.5.357.

BRTKO, J. 2022. Biological functions of kojic acid and its derivatives in medicine, cosmetics, and food industry: Insights into health aspects. *Archiv der Pharmazie,* 355**,** 2200215. DOI: https://doi.org/10.1002/ardp.202200215.

BRTKO, J., RONDAHL, L., FICKOVA, M., HUDECOVA, D., EYBL, V. & UHER, M. 2004. Kojic acid and its derivatives: history and present state of art. *Central european journal of public health,* 12**,** S16-S17.

BURNETT, C. L., BERGFELD, W. F., BELSITO, D. V., HILL, R. A., KLAASSEN, C. D., LIEBLER, D. C., MARKS, J. G., SHANK, R. C., SLAGA, T. J. & SNYDER, P. W. 2010a. Final report of the safety assessment of kojic acid as used in cosmetics. *International Journal of Toxicology,* 29**,** 244S-273S.

BURNETT, C. L., BERGFELD, W. F., BELSITO, D. V., HILL, R. A., KLAASSEN, C. D., LIEBLER, D. C., MARKS, J. G., SHANK, R. C., SLAGA, T. J., SNYDER, P. W. & ANDERSEN, F. A. 2010b. Final Report of the Safety Assessment of Kojic Acid as Used in Cosmetics. *International Journal of Toxicology,* 29**,** 244-273. DOI: 10.1177/1091581810385956.

BURNS, T., BREATHNACH, S. M., COX, N. & GRIFFITHS, C. 2008. *Rook's textbook of dermatology,* John Wiley & Sons.

CABANES, J., CHAZARRA, S. & GARCIA-CARMONA, F. 1994. Kojic acid, a cosmetic skin whitening agent, is a slow-binding inhibitor of catecholase activity of tyrosinase. *Journal of Pharmacy and Pharmacology,* 46**,** 982-985. DOI: 10.1111/j.2042-7158.1994.tb03253.x.

CARRICO, C., MEYER, J. G., HE, W., GIBSON, B. W. & VERDIN, E. 2018. The mitochondrial acylome emerges: proteomics, regulation by sirtuins, and metabolic and disease implications. *Cell metabolism,* 27**,** 497-512.

CATARZI, S., ROMAGNOLI, C., MARCUCCI, G., FAVILLI, F., IANTOMASI, T. & VINCENZINI, M. T. 2011. Redox regulation of ERK1/2 activation induced by sphingosine 1-phosphate in fibroblasts: involvement of NADPH oxidase and platelet-derived growth factor receptor. *Biochimica et Biophysica Acta (BBA)-General Subjects,* 1810**,** 446-456.

CHANG, T.-S. 2009. An Updated Review of Tyrosinase Inhibitors. *International Journal of Molecular Sciences,* 10**,** 2440-2475.

CHEN, J., LIU, Y., ZHAO, Z. & QIU, J. 2021. Oxidative stress in the skin: Impact and related protection. *International Journal of Cosmetic Science,* 43**,** 495-509. DOI: https://doi.org/10.1111/ics.12728.

CHEN, J. S., WEI, C. I. & MARSHALL, M. R. 1991. Inhibition mechanism of kojic acid on polyphenol oxidase. *Journal of Agricultural and Food Chemistry,* 39**,** 1897-1901. DOI: 10.1021/jf00011a001.

CHEN, Y.-H., LU, P.-J., HULME, C. & SHAW, A. Y. 2013. Synthesis of kojic acid-derived copper-chelating apoptosis inducing agents. *Medicinal Chemistry Research,* 22**,** 995-1003. DOI: 10.1007/s00044-012-0094-y.

CHEN, Y. Y., LIU, L. P., ZHOU, H., ZHENG, Y. W. & LI, Y. M. 2022. Recognition of Melanocytes in Immuno-Neuroendocrinology and Circadian Rhythms: Beyond the Conventional Melanin Synthesis. *Cells,* 11. DOI: 10.3390/cells11132082.

CHIB, S., JAMWAL, V. L., KUMAR, V., GANDHI, S. G. & SARAN, S. 2023. Fungal production of kojic acid and its industrial applications. *Applied Microbiology and Biotechnology,* 107**,** 2111-2130. DOI: 10.1007/s00253-023-12451-1.

CHOI, H., KIM, K., HAN, J., CHOI, H., JIN, S. H., LEE, E. K., SHIN, D. W., LEE, T. R., LEE, A.-Y. & NOH, M. 2012. Kojic acid-induced IL-6 production in human keratinocytes plays a role in its anti-melanogenic activity in skin. *Journal of Dermatological Science,* 66**,** 207-215. DOI: https://doi.org/10.1016/j.jdermsci.2012.03.002.

CHOUCHANI, E. T., PELL, V. R., GAUDE, E., AKSENTIJEVIĆ, D., SUNDIER, S. Y., ROBB, E. L., LOGAN, A., NADTOCHIY, S. M., ORD, E. N. & SMITH, A. C. 2014. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature,* 515**,** 431-435.

CHOWDHRY, S., ZHANG, Y., MCMAHON, M., SUTHERLAND, C., CUADRADO, A. & HAYES, J. D. 2013. Nrf2 is controlled by two distinct β-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity. *Oncogene,* 32**,** 3765-3781.

CICHOREK, M., WACHULSKA, M., STASIEWICZ, A. & TYMIŃSKA, A. 2013. Review paperSkin melanocytes: biology and development. *Advances in Dermatology and Allergology/Postępy Dermatologii i Alergologii,* 30**,** 30-41. DOI: 10.5114/pdia.2013.33376.

COLEMAN, M. L., MARSHALL, C. J. & OLSON, M. F. 2004. RAS and RHO GTPases in G1-phase cell-cycle regulation. *Nature reviews Molecular cell biology,* 5**,** 355-366.

COOKSON, B. T. & BRENNAN, M. A. 2001. Pro-inflammatory programmed cell death. *Trends in microbiology,* 9**,** 113-114.

COUPLAND, K. & NIEHAUS, W. G. 1987. Effect of nitrogen supply, Zn2+, and salt concentration on kojic acid and versicolorin biosynthesis by *Aspergillus parasiticus*. *Experimental Mycology,* 11**,** 206-213. DOI: https://doi.org/10.1016/0147-5975(87)90006-5.

CRIOLLO, A., SENOVILLA, L., AUTHIER, H., MAIURI, M. C., MORSELLI, E., VITALE, I., KEPP, O., TASDEMIR, E., GALLUZZI, L. & SHEN, S. 2010. The IKK complex contributes to the induction of autophagy. *The EMBO journal,* 29**,** 619-631.

CUENDA, A. & ROUSSEAU, S. 2007. p38 MAP-kinases pathway regulation, function and role in human diseases. *Biochimica et Biophysica Acta (BBA)* 1773**,** 1358-75. DOI: 10.1016/j.bbamcr.2007.03.010.

CUENDA, A. & SANZ-EZQUERRO, J. J. 2017. p38γ and p38δ: From Spectators to Key Physiological Players. *Trends in Biochemical Sciences,* 42**,** 431-442. DOI: 10.1016/j.tibs.2017.02.008.

DA COSTA, J. P., RODRIGUES, A. P. D., FARIAS, L. H. S., FRADE, P. C. R., DA SILVA, B. J. M., DO NASCIMENTO, J. L. M. & SILVA, E. O. 2018. Biological effects of kojic acid on human monocytes in vitro. *Biomedicine & Pharmacotherapy,* 101**,** 100-106.

DATTA, S. R., BRUNET, A. & GREENBERG, M. E. 1999. Cellular survival: a play in three Akts. *Genes & Development,* 13**,** 2905-2927.

DE CALDAS FELIPE, M. T., DO NASCIMENTO BARBOSA, R., BEZERRA, J. D. P. & DE SOUZA-MOTTA, C. M. 2023. Production of kojic acid by Aspergillus species: Trends and applications. *Fungal Biology Reviews,* 45**,** 100313.

DERI, B., KANTEEV, M., GOLDFEDER, M., LECINA, D., GUALLAR, V., ADIR, N. & FISHMAN, A. 2016. The unravelling of the complex pattern of tyrosinase inhibition. *Scientific reports,* 6**,** 1-10. DOI: 10.1038/srep34993.

DÉRIJARD, B., RAINGEAUD, J., BARRETT, T., WU, I.-H., HAN, J., ULEVITCH, R. J. & DAVIS, R. J. 1995. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science,* 267**,** 682-685.

DESAI, S., AYRES, E., BAK, H., GREEN, D. & ZHENG, Q. 2019. Effect of a tranexamic acid, kojic acid, and niacinamide containing serum on facial dyschromia: a clinical evaluation. *Journal of Drugs in Dermatology,* 18**,** 454-459.

DHAR, A., YOUNG, M. R. & COLBURN, N. H. 2002. The role of AP-1, NF-κB and ROS/NOS in skin carcinogenesis: the JB6 model is predictive. *Molecular and cellular biochemistry,* 234**,** 185-193.

DINKOVA-KOSTOVA, A. T., HOLTZCLAW, W. D., COLE, R. N., ITOH, K., WAKABAYASHI, N., KATOH, Y., YAMAMOTO, M. & TALALAY, P. 2002. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proceedings of the National Academy of Sciences,* 99**,** 11908-11913.

DOZA, Y. N., CUENDA, A., THOMAS, G. M., COHEN, P. & NEBREDA, A. R. 1995. Activation of the MAP kinase homologue RK requires the phosphorylation of Thr-180 and Tyr-182 and both residues are phosphorylated in chemically stressed KB cells. *FEBS letters,* 364**,** 223-228.

DRAELOS, Z. D., YATSKAYER, M., BHUSHAN, P., PILLAI, S. & ORESAJO, C. 2010. Evaluation of a kojic acid, emblica extract, and glycolic acid formulation compared with hydroquinone 4% for skin lightening. *Cutis,* 86**,** 153-8.

DUCHEN, M. R. 2004. Roles of Mitochondria in Health and Disease. *Diabetes,* 53**,** S96-S102. DOI: 10.2337/diabetes.53.2007.S96.

EL-KADY, I. A., ZOHRI, A. N. A. & HAMED, S. R. 2014. Kojic acid production from agro-industrial by-products using fungi. *Biotechnology Research International,* 2014**,** 1-10.

EL-METWALLY, M. M., ELBEALY, E. R., BELTAGY, D. M., SHAABAN, M. & EL-KOTT, A. F. 2020. Suppressive efficiency of Kojic acid from Aspergillus tamarii MM11 against HepG-2 cell line derived from human liver cancer. *Tropical Journal of Pharmaceutical Research,* 19**,** 1661-1668.

ESPINOSA-DIEZ, C., MIGUEL, V., MENNERICH, D., KIETZMANN, T., SÁNCHEZ-PÉREZ, P., CADENAS, S. & LAMAS, S. 2015. Antioxidant responses and cellular adjustments to oxidative stress. *Redox Biology,* 6**,** 183-197. DOI: https://doi.org/10.1016/j.redox.2015.07.008.

FAN, D., YANG, Z., LIU, F.-Y., JIN, Y.-G., ZHANG, N., NI, J., YUAN, Y., LIAO, H.-H., WU, Q.-Q. & XU, M. 2018. Sesamin protects against cardiac remodeling via Sirt3/ROS pathway. *Cellular Physiology and Biochemistry,* 44**,** 2212-2227.

FANG, S., CHEN, L., YU, M., CHENG, B., LIN, Y., MORRIS-NATSCHKE, S. L., LEE, K.-H., GU, Q. & XU, J. 2015. Synthesis, antitumor activity, and mechanism of action of 6-acrylic phenethyl ester-2-pyranone derivatives. *Organic & Biomolecular Chemistry,* 13**,** 4714-4726. DOI: 10.1039/C5OB00007F.

FARAGE, M. A., MILLER, K. W. & MAIBACH, H. I. 2009. *Textbook of aging skin,* Springer Science & Business Media.

FELIPE, M. T. D. C., BARBOSA, R. D. N., BEZERRA, J. D. P. & SOUZA-MOTTA, C. M. D. 2023. Production of kojic acid by Aspergillus species: Trends and applications. *Fungal Biology Reviews,* 45**,** 100313. DOI: https://doi.org/10.1016/j.fbr.2023.100313.

FETTER, T., DE GRAAF, D. M., CLAUS, I. & WENZEL, J. 2023. Aberrant inflammasome activation as a driving force of human autoimmune skin disease. *Frontiers in Immunology,* 14. DOI: 10.3389/fimmu.2023.1190388.

FINDLAY, G. H., MORRISON, J. G. & SIMSON, I. W. 1975. Exogenous ochronosis and pigmented colloid milium from hydroquinone bleaching creams. *British Journal of Dermatology,* 93**,** 613-622. DOI: 10.1111/j.1365-2133.1975.tb05110.x.

FINLEY, L. W., CARRACEDO, A., LEE, J., SOUZA, A., EGIA, A., ZHANG, J., TERUYA-FELDSTEIN, J., MOREIRA, P. I., CARDOSO, S. M. & CLISH, C. B. 2011. SIRT3 opposes reprogramming of cancer cell metabolism through HIF1α destabilization. *Cancer cell,* 19**,** 416-428.

FLOHR, C. & HAY, R. 2021. Putting the burden of skin diseases on the global map. *British Journal of Dermatology,* 184**,** 189-190. DOI: https://doi.org/10.1111/bjd.19704.

FRANKLIN, B. S., BOSSALLER, L., DE NARDO, D., RATTER, J. M., STUTZ, A., ENGELS, G., BRENKER, C., NORDHOFF, M., MIRANDOLA, S. R. & AL-AMOUDI, A. 2014. The adaptor ASC has extracellular and'prionoid'activities that propagate inflammation. *Nature immunology,* 15**,** 727-737.

FRITZ, J. H., FERRERO, R. L., PHILPOTT, D. J. & GIRARDIN, S. E. 2006. Nod-like proteins in immunity, inflammation and disease. *Nature immunology,* 7**,** 1250-1257.

GIBELLINI, L., PINTI, M., BERETTI, F., PIERRI, C. L., ONOFRIO, A., RICCIO, M., CARNEVALE, G., BIASI, S., MILENA, N., TORELLI, F., BORALDI, F., POL, A. & COSSARIZZA, A. 2014. Sirtuin 3 interacts with Lon protease and regulates its acetylation state. *Mitochondrion,* 18**,** 76-81. DOI: 10.1016/j.mito.2014.08.001.

GILLBRO, J. M. & OLSSON, M. J. 2011. The melanogenesis and mechanisms of skin-lightening agents – existing and new approaches. *International Journal of Cosmetic Science,* 33**,** 210-221. DOI: 10.1111/j.1468-2494.2010.00616.x.

GROSLAMBERT, M. & PY, B. F. 2018. Spotlight on the NLRP3 inflammasome pathway. *Journal of Inflammation Research,* 11**,** 359-374. DOI: 10.2147/jir.S141220.

GUO, Y. J., PAN, W. W., LIU, S. B., SHEN, Z. F., XU, Y. & HU, L. L. 2020. ERK/MAPK signalling pathway and tumorigenesis (Review). *Experimental and Therapeutic Medicine,* 19**,** 1997-2007. DOI: 10.3892/etm.2020.8454.

GUPTA, A. K., GOVER, M. D., NOURI, K. & TAYLOR, S. 2006. The treatment of melasma: a review of clinical trials. *Journal of the American Academy of Dermatology,* 55**,** 1048-65. DOI: 10.1016/j.jaad.2006.02.009.

GURUNG, P., ANAND, P. K., MALIREDDI, R., VANDE WALLE, L., VAN OPDENBOSCH, N., DILLON, C. P., WEINLICH, R., GREEN, D. R., LAMKANFI, M. & KANNEGANTI, T.-D. 2014. FADD and caspase-8 mediate priming and activation of the canonical and noncanonical Nlrp3 inflammasomes. *The Journal of Immunology,* 192**,** 1835-1846.

HANSON, P. I. & WHITEHEART, S. W. 2005. AAA+ proteins: have engine, will work. *Nature reviews Molecular cell biology,* 6**,** 519-529.

HASIL, A., MEHMOOD, A., NOUREEN, S. & AHMED, M. 2020. Experimental and theoretical charge density analysis of skin whitening agent kojic acid. *Journal of Molecular Structure,* 1216**,** 128295.

HAYES, J. D., DINKOVA-KOSTOVA, A. T. & TEW, K. D. 2020. Oxidative stress in cancer. *Cancer cell,* 38**,** 167-197.

HEBERT, A. S., DITTENHAFER-REED, K. E., YU, W., BAILEY, D. J., SELEN, E. S., BOERSMA, M. D., CARSON, J. J., TONELLI, M., BALLOON, A. J. & HIGBEE, A. J. 2013. Calorie

restriction and SIRT3 trigger global reprogramming of the mitochondrial protein acetylome. *Molecular cell,* 49**,** 186-199.

HEMMINGS, B. A. & RESTUCCIA, D. F. 2012. PI3K-PKB/Akt pathway. *Cold Spring Harbour Perspectives in Biology,* 4**,** a011189. DOI: 10.1101/cshperspect.a011189.

HIRA, Y., OHYAMA, Y., KAIMI, K., MISHIMA, Y. & FURUKAWA, T. 1985. Inhibitory effect of kojic acid contained cream in UV-induced pigmentationin human skin. *Japanese Cosmetic Science Society,* 9**,** 109.

HORNUNG, V., BAUERNFEIND, F., HALLE, A., SAMSTAD, E. O., KONO, H., ROCK, K. L., FITZGERALD, K. A. & LATZ, E. 2008. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nature immunology,* 9**,** 847-856.

HWA, C., BAUER, E. A. & COHEN, D. E. 2011. Skin biology. *Dermatologic Therapy,* 24**,** 464-470. DOI: https://doi.org/10.1111/j.1529-8019.2012.01460.x.

IARC Kojic Acid. *International Agency into Research on Cancer Monographs,* 79**,** 607-618.

ICHIMOTO, I., FUJII, K. & TATSUMI, C. 1965. Studies on Kojic Acid and its Related γ-Pyrone Compounds. *Agricultural and Biological Chemistry,* 29**,** 325-330. DOI: 10.1080/00021369.1965.10858386.

ISKANDAR, I. Y. K., PARISI, R., GRIFFITHS, C. E. M., ASHCROFT, D. M. & ATLAS, T. G. P. 2021. Systematic review examining changes over time and variation in the incidence and prevalence of psoriasis by age and gender*. *British Journal of Dermatology,* 184**,** 243-258. DOI: https://doi.org/10.1111/bjd.19169.

ISRAËL, A. 2010. The IKK complex, a central regulator of NF-κB activation. *Cold Spring Harbor perspectives in biology,* 2**,** a000158.

ITOH, K., CHIBA, T., TAKAHASHI, S., ISHII, T., IGARASHI, K., KATOH, Y., OYAKE, T., HAYASHI, N., SATOH, K. & HATAYAMA, I. 1997. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochemical and biophysical research communications,* 236**,** 313-322.

JAIN, A. K., MAHAJAN, S. & JAISWAL, A. K. 2008. Phosphorylation and dephosphorylation of tyrosine 141 regulate stability and degradation of INrf2: a novel mechanism in Nrf2 activation. *Journal of Biological Chemistry,* 283**,** 17712-17720.

KARIN, M. 2006. Nuclear factor-kappaB in cancer development and progression. *Nature,* 441**,** 431-6. DOI: 10.1038/nature04870.

KARIN, M. & DELHASE, M. The IκB kinase (IKK) and NF-κB: key elements of proinflammatory signalling. Seminars in immunology, 2000. Elsevier, 85-98.

KHAN, A., PARK, T. J., IKRAM, M., AHMAD, S., AHMAD, R., JO, M. G. & KIM, M. O. 2021. Antioxidative and Anti-inflammatory Effects of Kojic Acid in Aβ-Induced Mouse Model of Alzheimer's Disease. *Molecular Neurobiology,* 58**,** 5127-5140. DOI: 10.1007/s12035-021-02460-4.

KHEZRI, K., SAEEDI, M., MORTEZA-SEMNANI, K., AKBARI, J. & ROSTAMKALAEI, S. S. 2020. An emerging technology in lipid research for targeting hydrophilic drugs to the skin in the treatment of hyperpigmentation disorders: kojic acid-solid lipid nanoparticles. *Artificial Cells, Nanomedicine, and Biotechnology,* 48**,** 841-853. DOI: 10.1080/21691401.2020.1770271.

KIM, D. H., HWANG, J. S., BAEK, H. S., KIM, K. J., LEE, B. G., CHANG, I., KANG, H. H. & LEE, O. S. 2003. Development of 5-[(3-aminopropyl)phosphinooxy]-2-(hydroxymethyl)-4H-pyran-4-one as a novel whitening agent. *Chemical and Pharmaceutical Bulletin* 51**,** 113-116. DOI: 10.1248/cpb.51.113.

KIM, J.-H., HONG, S.-K., WU, P.-K., RICHARDS, A. L., JACKSON, W. T. & PARK, J.-I. 2014. Raf/MEK/ERK can regulate cellular levels of LC3B and SQSTM1/p62 at expression levels. *Experimental cell research,* 327**,** 340-352.

KIM, J. H., CAMPBELL, B. C., CHAN, K. L., MAHONEY, N. & HAFF, R. P. 2013a. Synergism of Antifungal Activity between Mitochondrial Respiration Inhibitors and Kojic Acid. *Molecules,* 18**,** 1564-1581.

KIM, J. H., CHANG, P.-K., CHAN, K. L., FARIA, N. C. G., MAHONEY, N., KIM, Y. K., MARTINS, M. D. L. & CAMPBELL, B. C. 2012. Enhancement of commercial antifungal agents by Kojic Acid. *International Journal of Molecular Sciences,* 13**,** 13867-13880. DOI: 10.3390/ijms131113867.

KIM, S.-U., PARK, Y.-H., MIN, J.-S., SUN, H.-N., HAN, Y.-H., HUA, J.-M., LEE, T.-H., LEE, S.-R., CHANG, K.-T. & KANG, S. W. 2013b. Peroxiredoxin I is a ROS/p38 MAPK-dependent inducible antioxidant that regulates NF-κB-mediated iNOS induction and microglial activation. *Journal of Neuroimmunology,* 259**,** 26-36.

KONG, T., LIU, M., JI, B., BAI, B., CHENG, B. & WANG, C. 2019. Role of the Extracellular Signal-Regulated Kinase 1/2 Signaling Pathway in Ischemia-Reperfusion Injury. *Frontiers in Physiology,* 10. DOI: 10.3389/fphys.2019.01038.

KOOYERS, T. J. & WESTERHOF, W. 2004. Toxicological aspects and health risks associated with hydroquinone in skin bleaching formula. *Nederlands Tijdschrift voor Geneeskunde,* 148**,** 768-771.

KOTYZOVA, D., EYBL, V., KOUTENSKY, J., BRTKO, J. & GLATTRE, E. 2004. Effects of kojic acid on oxidative damage and on iron and trace element level in iron-overloaded mice and rats. *Central European Journal of Public Health,* 12 Suppl**,** 41-44.

KUMAR, K. S., VANI, M. G., WANG, S. Y., LIAO, J. W., HSU, L. S., YANG, H. L. & HSEU, Y. C. 2013. In vitro and in vivo studies disclosed the depigmenting effects of gallic acid: A novel skin lightening agent for hyperpigmentary skin diseases. *Biofactors,* 39**,** 259-270.

KUMARI, P., GUPTA, S., NARAYANA, C., AHMAD, S., VISHNOI, N., SINGH, S. & SAGAR, R. 2018. Stereoselective synthesis of carbohydrate fused pyrano[3,2-c]pyranones as anticancer agents. *New Journal of Chemistry,* 42**,** 13985-13997. DOI: 10.1039/C8NJ01395K.

KWON, S.-H., HWANG, Y.-J., LEE, S.-K. & PARK, K.-C. 2016. Heterogeneous pathology of melasma and its clinical implications. *International journal of molecular sciences,* 17**,** 824.

LAJIS, A. F. B., HAMID, M. & ARIFF, A. B. 2012a. Depigmenting effect of Kojic acid esters in hyperpigmented B16F1 melanoma cells. *Journal of Biomedicine & Biotechnology,* 2012**,** 1-9. DOI: 10.1155/2012/952452.

LAJIS, A. F. B., HAMID, M. & ARIFF, A. B. 2012b. Depigmenting Effect of Kojic Acid Esters in Hyperpigmented B16F1 Melanoma Cells. *Journal of Biomedicine and Biotechnology,* 2012**,** 952452. DOI: 10.1155/2012/952452.

LAUGHTER, M., MAYMONE, M., MASHAYEKHI, S., ARENTS, B., KARIMKHANI, C., LANGAN, S., DELLAVALLE, R. & FLOHR, C. 2021. The global burden of atopic dermatitis: lessons from the Global Burden of Disease Study 1990–2017. *British Journal of Dermatology,* 184**,** 304-309.

LAWRENCE, T. 2009. The nuclear factor NF-κB pathway in inflammation. *Cold Spring Harbor perspectives in biology,* 1**,** a001651.

LAYTON, A. M., THIBOUTOT, D. & TAN, J. 2021. Reviewing the global burden of acne: how could we improve care to reduce the burden?*. *British Journal of Dermatology,* 184**,** 219-225. DOI: https://doi.org/10.1111/bjd.19477.

LEE, H. F., BOLTJES, B. & EISENMAN, W. 1950. Kojic Acid as an Inhibitor of Tubercle Bacilli. *American Review of Tuberculosis and Pulmonary Diseases,* 61**,** 738-741.

LEE, I. & SUZUKI, C. K. 2008. Functional mechanics of the ATP-dependent Lon protease- lessons from endogenous protein and synthetic peptide substrates. *Biochimica et Biophysica Acta (BBA),* 1784**,** 727-35. DOI: 10.1016/j.bbapap.2008.02.010.

LEE, M., RHO, H. S. & CHOI, K. 2019. Anti-inflammatory effects of a P-coumaric acid and kojic acid derivative in LPS-stimulated RAW264. 7 macrophage cells. *Biotechnology and Bioprocess Engineering,* 24**,** 653-657.

LENNICKE, C. & COCHEMÉ, H. M. 2021. Redox metabolism: ROS as specific molecular regulators of cell signaling and function. *Molecular Cell,* 81**,** 3691-3707.

LI, T.-X., LIANG, J.-X., LIU, L.-L., SHI, F.-C., JIA, X.-W., LI, M.-H. & XU, C.-P. 2021. Novel kojic acid derivatives with anti-inflammatory effects from Aspergillus versicolor. *Fitoterapia,* 154**,** 105027.

LIM, J. T. 1999. Treatment of melasma using kojic acid in a gel containing hydroquinone and glycolic acid. *Dermatologic Surgery,* 25**,** 282-4. DOI: 10.1046/j.1524-4725.1999.08236.x.

LIN, T.-Y., TSAI, M.-C., TU, W., YEH, H.-C., WANG, S.-C., HUANG, S.-P. & LI, C.-Y. 2021. Role of the NLRP3 Inflammasome: Insights Into Cancer Hallmarks. *Frontiers in Immunology,* 11. DOI: 10.3389/fimmu.2020.610492.

LIU, T., ZHANG, L., JOO, D. & SUN, S.-C. 2017. NF-κB signaling in inflammation. *Signal Transduction and Targeted Therapy,* 2**,** 17023. DOI: 10.1038/sigtrans.2017.23.

LU, Y., ZHU, W. Y., TAN, C., YU, G. H. & GU, J. X. 2002. Melanocytes are potential immunocompetent cells: evidence from recognition of immunological characteristics of cultured human melanocytes. *Pigment Cell and Melanoma Research,* 15**,** 454-60. DOI: 10.1034/j.1600-0749.2002.02065.x.

MA, D.-L., XU, T., CHAN, D. S.-H., MAN, B. Y.-W., FONG, W.-F. & LEUNG, C.-H. 2011. A highly selective, label-free, homogenous luminescent switch-on probe for the detection of nanomolar transcription factor NF-kappaB. *Nucleic acids research,* 39**,** e67-e67.

MAILLOUX, R. J. 2020. An Update on Mitochondrial Reactive Oxygen Species Production. *Antioxidants,* 9**,** 472.

MANN, T., GERWAT, W., BATZER, J., EGGERS, K., SCHERNER, C., WENCK, H., STÄB, F., HEARING, V. J., RÖHM, K.-H. & KOLBE, L. 2018. Inhibition of human tyrosinase requires molecular motifs distinctively different from mushroom tyrosinase. *Journal of Investigative Dermatology,* 138**,** 1601-1608.

MARINHO, H. S., REAL, C., CYRNE, L., SOARES, H. & ANTUNES, F. 2014. Hydrogen peroxide sensing, signaling and regulation of transcription factors. *Redox biology,* 2**,** 535-562.

MARSHALL, C. J. 1994. MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Current opinion in genetics & development,* 4**,** 82-89.

MARTÍNEZ-LIMÓN, A., JOAQUIN, M., CABALLERO, M., POSAS, F. & DE NADAL, E. 2020. The p38 Pathway: From Biology to Cancer Therapy. *International Journal of Molecular Sciences,* 21**,** 1913.

MATZINGER, P. 1994. Tolerance, danger, and the extended family. *Annual review of immunology,* 12**,** 991-1045.

MAYER, A. M. 1986. Polyphenol oxidases in plants-recent progress. *Phytochemistry,* 26**,** 11-20. DOI: https://doi.org/10.1016/S0031-9422(00)81472-7.

MCMAHON, M., THOMAS, N., ITOH, K., YAMAMOTO, M. & HAYES, J. D. 2004. Redox-regulated turnover of Nrf2 is determined by at least two separate protein domains, the redox-sensitive Neh2 degron and the redox-insensitive Neh6 degron. *Journal of Biological Chemistry,* 279**,** 31556-31567.

MEDZHITOV, R. & JANEWAY, C. A. 1997. Innate immunity: the virtues of a nonclonal system of recognition. *Cell,* 91**,** 295-298.

MEYLAN, E., TSCHOPP, J. & KARIN, M. 2006. Intracellular pattern recognition receptors in the host response. *Nature,* 442**,** 39-44.

MICHAN, S. & SINCLAIR, D. 2007. Sirtuins in mammals: insights into their biological function. *Biochemical Journal,* 404**,** 1-13. DOI: 10.1042/bj20070140.

MISHIMA, Y., SHIBATA, T., SETO, H., OHYAMA, Y. & HATAE, S. 1994. Inhibitory action of kojic acid on melanogensis and its therapeutic effect for various human hyper-pigmentation disorders. *Skin research,* 36**,** 134-150.

MITCHELL, J. A., PAUL-CLARK, M. J., CLARKE, G. W., MCMASTER, S. K. & CARTWRIGHT, N. 2007. Critical role of toll-like receptors and nucleotide oligomerisation domain in the regulation of health and disease. *Journal of Endocrinology,* 193**,** 323-330.

MOHAMAD, R., MOHAMED, M., SUHAILI, N., SALLEH, M. & ARIFF, A. 2010. Kojic acid: Applications and development of fermentation process for production. *Biotechnology and Molecular Biology Reviews,* 5**,** 24-37.

MOLONEY, J. N. & COTTER, T. G. 2018. ROS signalling in the biology of cancer. *Seminars in Cell & Developmental Biology,* 80**,** 50-64. DOI: https://doi.org/10.1016/j.semcdb.2017.05.023.

MOON, K. Y., AHN, K. S., LEE, J. & KIM, Y. S. 2001. Kojic acid, a potential inhibitor of NF-kappaB activation in transfectant human HaCaT and SCC-13 cells. *Archives of Pharmacal Research,* 24**,** 307-311. DOI: 10.1007/bf02975097.

MURAKAMI, T., OCKINGER, J., YU, J., BYLES, V., MCCOLL, A., HOFER, A. M. & HORNG, T. 2012. Critical role for calcium mobilization in activation of the NLRP3 inflammasome. *Proceedings of the National Academy of Sciences,* 109**,** 11282-11287.

MURPHREE, R. W. 2017. Impairments in Skin Integrity. *Nursing Clinics of North America,* 52**,** 405-417. DOI: 10.1016/j.cnur.2017.04.008.

NAKAGAWA, M., KAWAI, K. & KAWAI, K. 1995. Contact allergy to kojic acid in skin care products. *Contact Dermatitis,* 32**,** 9-13. DOI: 10.1111/j.1600-0536.1995.tb00832.x.

NAKAYAMA, H. 1982. Treatment of chloasma with topical Kojic Acid. *Japanese Journal of Clinical Dermatology,* 36**,** 715-722.

NAKHI, A., ADEPU, R., RAMBABU, D., KISHORE, R., VANAJA, G. R., KALLE, A. M. & PAL, M. 2012. Thieno[3,2-c]pyran-4-one based novel small molecules: Their synthesis, crystal structure analysis and in vitro evaluation as potential anticancer agents. *Bioorganic & Medicinal Chemistry Letters,* 22**,** 4418-4427. DOI: https://doi.org/10.1016/j.bmcl.2012.04.109.

NAWARAK, J., HUANG-LIU, R., KAO, S.-H., LIAO, H.-H., SINCHAIKUL, S., CHEN, S.-T. & CHENG, S.-L. 2008. Proteomics Analysis of Kojic Acid Treated A375 Human Malignant Melanoma Cells. *Journal of Proteome Research,* 7**,** 3737-3746. DOI: 10.1021/pr7008737.

NEELI, P. K., GOLLAVILLI, P. N., MALLAPPA, S., HARI, S. G. & KOTAMRAJU, S. 2020. A novel metadherinΔ7 splice variant enhances triple negative breast cancer aggressiveness by modulating mitochondrial function via NFκB-SIRT3 axis. *Oncogene,* 39**,** 2088-2102.

NGO, J. K. & DAVIES, K. J. A. 2007. Importance of the Lon Protease in Mitochondrial Maintenance and the Significance of Declining Lon in Aging. *Annals of the New York Academy of Sciences,* 1119**,** 78-87. DOI: https://doi.org/10.1196/annals.1404.015.

NIDAI OZES, O., MAYO, L. D., GUSTIN, J. A., PFEFFER, S. R., PFEFFER, L. M. & DONNER, D. B. 1999. NF-κB activation by tumour necrosis factor requires the Akt serine–threonine kinase. *Nature,* 401**,** 82-85. DOI: 10.1038/43466.

NIWA, Y. & AKAMATSU, H. 1991. Kojic acid scavenges free radicals while potentiating leukocyte functions including free radical generation. *Inflammation,* 15**,** 303-315. DOI: 10.1007/BF00917315.

NOH, J.-M., KWAK, S.-Y., SEO, H., SEO, J.-H., KIM, B.-G. & LEE, Y.-S. 2009. Kojic acid-amino acid conjugates as tyrosinase inhibitors. *Bioorganic & medicinal chemistry letters,* 19**,** 5586-9. DOI: 10.1016/j.bmcl.2009.08.041.

NOH, J. M., KWAK, S. Y., KIM, D. H. & LEE, Y. S. 2007. Kojic acid-tripeptide amide as a new tyrosinase inhibitor. *Biopolymers,* 88**,** 300-7. DOI: 10.1002/bip.20670.

OECKINGHAUS, A. & GHOSH, S. 2009. The NF-κB family of transcription factors and its regulation. *Cold Spring Harbor perspectives in biology,* 1**,** a000034.

ONDROVICOVÁ, G., LIU, T., SINGH, K., TIAN, B., LI, H., GAKH, O., PEREČKO, D., JANATA, J. I., GRANOT, Z. & ORLY, J. 2005. Cleavage site selection within a folded substrate by the ATP-dependent lon protease. *Journal of Biological Chemistry,* 280**,** 25103-25110.

ORESAJO, C., YATSKAYER, M., FOLTIS, P. & HANSENNE, I. 2008. Comparative clinical assessment of two skin lightening products in female subjects with dyschromia. *Journal of the American Academy of Dermatology,* 58**,** AB26-AB26.

ORIOLI, D. & DELLAMBRA, E. 2018. Epigenetic Regulation of Skin Cells in Natural Aging and Premature Aging Diseases. *Cells,* 7. DOI: 10.3390/cells7120268.

OVERMEYER, J. H. & MALTESE, W. A. 2011. Death pathways triggered by activated Ras in cancer cells. *Frontiers in bioscience: a journal and virtual library,* 16**,** 1693.

PAP, M. & COOPER, G. M. 1998. Role of Glycogen Synthase Kinase-3 in the Phosphatidylinositol 3-Kinase/Akt Cell Survival Pathway*. *Journal of Biological Chemistry,* 273**,** 19929-19932. DOI: https://doi.org/10.1074/jbc.273.32.19929.

PARRISH, F. W., WILEY, B. J., SIMMONS, E. G. & LONG, L., JR. 1966. Production of aflatoxins and kojic acid by species of Aspergillus and Penicillium. *Applied microbiology,* 14**,** 139.

PASTORE, S., MASCIA, F., MARIOTTI, F., DATTILO, C., MARIANI, V. & GIROLOMONI, G. 2005. ERK1/2 Regulates Epidermal Chemokine Expression and Skin Inflammation1. *The Journal of Immunology,* 174**,** 5047-5056. DOI: 10.4049/jimmunol.174.8.5047.

PAWELEK, J. M. & KÖRNER, A. M. 1982. The biosynthesis of mammalian melanin. *American Science,* 70**,** 136-145.

PEKKARINEN, S. S., HEINONEN, I. M. & HOPIA, A. I. 1999. Flavonoids quercetin, myricetin, kaemferol and (+)-catechin as antioxidants in methyl linoleate. *Journal of the Science of Food and Agriculture,* 79**,** 499-506. DOI: 10.1002/(sici)1097-0010(19990315)79:4<499::Aid-jsfa204>3.0.Co;2-u.

PERDOMO, J., QUINTANA, C., GONZÁLEZ, I., HERNÁNDEZ, I., RUBIO, S., LORO, J. F., REITER, R. J., ESTÉVEZ, F. & QUINTANA, J. 2020. Melatonin Induces Melanogenesis in Human SK-MEL-1 Melanoma Cells Involving Glycogen Synthase Kinase-3 and Reactive Oxygen Species. *International Journal of Molecular Sciences,* 21. DOI: 10.3390/ijms21144970.

PEROKOVIĆ, V. P., CAR, Ž., USENIK, A., OPAČAK-BERNARDI, T., JURIĆ, A. & TOMIĆ, S. 2020. Adamantyl pyran-4-one derivatives and their in vitro antiproliferative activity. *Molecular Diversity,* 24**,** 253-263. DOI: 10.1007/s11030-019-09948-1.

PHASHA, V., SENABE, J., NDZOTOYI, P., OKOLE, B., FOUCHE, G. & CHUTURGOON, A. 2022. Review on the Use of Kojic Acid—A Skin-Lightening Ingredient. *Cosmetics,* 9**,** 64.

PLOTNIKOV, A., ZEHORAI, E., PROCACCIA, S. & SEGER, R. 2011. The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research,* 1813**,** 1619-1633.

POPOV, L.-D. 2020. Mitochondrial biogenesis: An update. *Journal of Cellular and Molecular Medicine,* 24**,** 4892-4899. DOI: https://doi.org/10.1111/jcmm.15194.

RASMEY, A.-H. M. & ABDEL-KAREEM, M. 2021. Optimization of culture conditions for Kojic acid production in surface fermentation by Aspergillus oryzae isolated from wheat grains. *Bulletin of Pharmaceutical Sciences. Assiut,* 44**,** 201-211.

REVATHIDEVI, S. & MUNIRAJAN, A. K. 2019. Akt in cancer: Mediator and more. *Seminars in Cancer Biology,* 59**,** 80-91. DOI: https://doi.org/10.1016/j.semcancer.2019.06.002.

RODRIGUES, A. P., FARIAS, L. H., CARVALHO, A. S., SANTOS, A. S., DO NASCIMENTO, J. L. & SILVA, E. O. 2014. A novel function for kojic acid, a secondary metabolite from Aspergillus fungi, as antileishmanial agent. *PLoS One,* 9**,** e91259. DOI: 10.1371/journal.pone.0091259.

RÜHL, S. & BROZ, P. 2015. Caspase-11 activates a canonical NLRP3 inflammasome by promoting K+ efflux. *European journal of immunology,* 45**,** 2927-2936.

SAEEDI, M., ESLAMIFAR, M. & KHEZRI, K. 2019. Kojic acid applications in cosmetic and pharmaceutical preparations. *Biomedicine and Pharmacotherapy,* 110**,** 582-593. DOI: 10.1016/j.biopha.2018.12.006.

SAEEDI, M., MORTEZA-SEMNANI, K., AKBARI, J., RAHIMNIA, S. M., AHMADI, F., CHOUBDARI, H., LOTFI, A. & HASHEMI, S. M. H. 2023. Development of kojic acid loaded collagen-chitosan nanoparticle as skin lightener product: In vitro and in vivo assessment. *Journal of Biomaterials Science, Polymer Edition***,** 1-17.

SAHA, R. N., JANA, M. & PAHAN, K. 2007. MAPK p38 regulates transcriptional activity of NF-kappaB in primary human astrocytes via acetylation of p65. *Journal of Immunology,* 179**,** 7101-9. DOI: 10.4049/jimmunol.179.10.7101.

SAITO, K. 1907. Über die Säurebildung bei Aspergillus Oryzœ Vorläufige Mittheilung [On the formation of acid in Aspergillus Oryzae - Preliminary communication.]. *Shokubutsugaku Zasshi,* 21**,** 7-11.

SARBASSOV, D. D., GUERTIN, D. A., ALI, S. M. & SABATINI, D. M. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science,* 307**,** 1098-1101.

SATTERSTROM, F. K., SWINDELL, W. R., LAURENT, G., VYAS, S., BULYK, M. L. & HAIGIS, M. C. 2015. Nuclear respiratory factor 2 induces SIRT 3 expression. *Aging Cell,* 14**,** 818-825.

SCCP 2021. Opinion on Kojic acid. *European Commission***,** 1-85.

SCHIEBER, M. & CHANDEL, NAVDEEP S. 2014. ROS Function in Redox Signaling and Oxidative Stress. *Current Biology,* 24**,** R453-R462. DOI: https://doi.org/10.1016/j.cub.2014.03.034.

SCHWER, B. & VERDIN, E. 2008a. Conserved metabolic regulatory functions of sirtuins. *Cell Metabolism,* 7**,** 104-12. DOI: 10.1016/j.cmet.2007.11.006.

SCHWER, B. & VERDIN, E. 2008b. Conserved metabolic regulatory functions of sirtuins. *Cell metabolism,* 7**,** 104-112.

SHE, S., JIANG, L., ZHANG, Z., YANG, M., HU, H., HU, P., LIAO, Y., YANG, Y. & REN, H. 2018. Identification of the C-Reactive Protein Interaction Network Using a Bioinformatics Approach Provides Insights into the Molecular Pathogenesis of Hepatocellular Carcinoma. *Cellular Physiology and Biochemistry,* 48**,** 741-752. DOI: 10.1159/000491903.

SIES, H., BERNDT, C. & JONES, D. P. 2017. Oxidative Stress. *Annual Review of Biochemistry,* 86**,** 715-748. DOI: 10.1146/annurev-biochem-061516-045037.

SINGH, F. V., CHAURASIA, S., JOSHI, M. D., SRIVASTAVA, A. K. & GOEL, A. 2007. Synthesis and in vivo antihyperglycemic activity of nature-mimicking furanyl-2-pyranones in STZ-S model. *Bioorganic & Medicinal Chemistry Letters,* 17**,** 2425-2429. DOI: https://doi.org/10.1016/j.bmcl.2007.02.036.

SIVAPRASAD, U. & BASU, A. 2008. Inhibition of ERK attenuates autophagy and potentiates tumour necrosis factor-α-induced cell death in MCF-7 cells. *Journal of cellular and molecular medicine,* 12**,** 1265-1271.

SLOMINSKI, A., TOBIN, D. J., SHIBAHARA, S. & WORTSMAN, J. 2004. Melanin Pigmentation in Mammalian Skin and Its Hormonal Regulation. *Physiological Reviews,* 84**,** 1155-1228. DOI: 10.1152/physrev.00044.2003.

SOMEYA, S., YU, W., HALLOWS, W. C., XU, J., VANN, J. M., LEEUWENBURGH, C., TANOKURA, M., DENU, J. M. & PROLLA, T. A. 2010. Sirt3 mediates reduction of oxidative damage and prevention of age-related hearing loss under caloric restriction. *Cell,* 143**,** 802-812.

SOUZA, M. D. L. M. D., SULYOK, M., FREITAS-SILVA, O., COSTA, S. S., BRABET, C., MACHINSKI JUNIOR, M., SEKIYAMA, B. L., VARGAS, E. A., KRSKA, R. & SCHUHMACHER, R. 2013. Cooccurrence of Mycotoxins in Maize and Poultry Feeds from Brazil by Liquid Chromatography/Tandem Mass Spectrometry. *The Scientific World Journal,* 2013**,** 427369. DOI: 10.1155/2013/427369.

SPORN, M. B. & LIBY, K. T. 2012. NRF2 and cancer: the good, the bad and the importance of context. *Nature Reviews Cancer,* 12**,** 564-571.

SRIRAM, G., BIGLIARDI, P. L. & BIGLIARDI-QI, M. 2015. Fibroblast heterogeneity and its implications for engineering organotypic skin models in vitro. *European Journal of Cell Biology,* 94**,** 483-512. DOI: 10.1016/j.ejcb.2015.08.001.

STEIN, L. R. & IMAI, S.-I. 2012. The dynamic regulation of NAD metabolism in mitochondria. *Trends in Endocrinology & Metabolism,* 23**,** 420-428. DOI: 10.1016/j.tem.2012.06.005.

SUN, S.-C., CHANG, J.-H. & JIN, J. 2013. Regulation of nuclear factor-κB in autoimmunity. *Trends in immunology,* 34**,** 282-289.

SUN, S.-C. & LEY, S. C. 2008. New insights into NF-κB regulation and function. *Trends in immunology,* 29**,** 469-478.

SUN, S. C. 2012. The noncanonical NF-κB pathway. *Immunological reviews,* 246**,** 125-140.

SUNDARESAN, N. R., BINDU, S., PILLAI, V. B., SAMANT, S., PAN, Y., HUANG, J.-Y., GUPTA, M., NAGALINGAM, R. S., WOLFGEHER, D. & VERDIN, E. 2015. SIRT3 blocks aging-associated tissue fibrosis in mice by deacetylating and activating glycogen synthase kinase 3β. *Molecular and cellular biology*.

SUR, I., ULVMAR, M. & TOFTGÅRD, R. 2008. The two-faced NF-kappaB in the skin. *International Reviews of Immunology,* 27**,** 205-23. DOI: 10.1080/08830180802130319.

SURYADI, H., IRIANTI, M. I. & SEPTIARINI, T. H. 2022. Methods of random mutagenesis of Aspergillus strain for increasing kojic acid production. *Current Pharmaceutical Biotechnology,* 23**,** 486-494.

SUTHIRAM, K. T., GHAZI, T., ABDUL, N. S. & CHUTURGOON, A. A. 2023. Kojic acid induces oxidative stress and anti-inflammatory effects in human hepatocellular carcinoma (HepG2) cells. *Toxicon,* 232**,** 107221. DOI: 10.1016/j.toxicon.2023.107221.

SUTTERWALA, F. S., HAASKEN, S. & CASSEL, S. L. 2014. Mechanism of NLRP3 inflammasome activation. *Annals of the New York Academy of Sciences,* 1319**,** 82-95.

TALEBI, M., MAJIDI, K., BASSAM, K., ABDI, M., DANESHVAR, M., MOAYEDI, S. S., POURHESABI, S., ATTARROSHAN, M., BOUMI, S., KABIRI, M., HOSSEINI, F. S., KHOSHNEVISZADEH, M. & AMANLOU, M. 2022. Synthesis, biological evaluation, and molecular docking analysis of novel 1, 3, 4-thiadiazole -based kojic acid derivatives as tyrosinase inhibitors. *Journal of Molecular Structure,* 1268**,** 133707. DOI: https://doi.org/10.1016/j.molstruc.2022.133707.

TETALI, B., FAHS, F. M. & MEHREGAN, D. 2020. Popular over-the-counter cosmeceutical ingredients and their clinical efficacy. *International Journal of Dermatology,* 59**,** 393-405. DOI: https://doi.org/10.1111/ijd.14718.

TORRENS-MAS, M., HERNÁNDEZ-LÓPEZ, R., PONS, D.-G., ROCA, P., OLIVER, J. & SASTRE-SERRA, J. 2019. Sirtuin 3 silencing impairs mitochondrial biogenesis and metabolism in colon cancer cells. *American Journal of Physiology-Cell Physiology,* 317**,** C398-C404. DOI: 10.1152/ajpcell.00112.2019.

TRIANTAFILOU, K., HUGHES, T. R., TRIANTAFILOU, M. & MORGAN, B. P. 2013. The complement membrane attack complex triggers intracellular Ca2+ fluxes leading to NLRP3 inflammasome activation. *Journal of cell science,* 126**,** 2903-2913.

UHER, M., KONECNY, V. & RAJNIAKOVA, O. 1994. Synthesis of 5-hydroxy-2-hydroxymethyl-4H-pyran-4-one derivatives with pesticide activity. *Chemical Papers,* 48**,** 282-284.

VAN TRAN, V., NGUYEN, T. L., MOON, J.-Y. & LEE, Y.-C. 2019. Core-shell materials, lipid particles and nanoemulsions, for delivery of active anti-oxidants in cosmetics applications: Challenges and development strategies. *Chemical Engineering Journal,* 368**,** 88-114.

VOOS, W. & POLLECKER, K. 2020. The Mitochondrial Lon Protease: Novel Functions off the Beaten Track? *Biomolecules,* 10. DOI: 10.3390/biom10020253.

WAKABAYASHI, N., ITOH, K., WAKABAYASHI, J., MOTOHASHI, H., NODA, S., TAKAHASHI, S., IMAKADO, S., KOTSUJI, T., OTSUKA, F. & ROOP, D. R. 2003. Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. *Nature genetics,* 35**,** 238-245.

WANG, M. & LIN, H. 2021. Understanding the Function of Mammalian Sirtuins and Protein Lysine Acylation. *Annual Review of Biochemistry,* 90**,** 245-285. DOI: 10.1146/annurev-biochem-082520-125411.

WAWRZYK-BOCHENEK, I., RAHNAMA, M., STACHURA, M., WILCZYŃSKI, S. & WAWRZYK, A. 2023. Evaluation of the Reduction of Skin Hyperpigmentation Changes under the Influence of a Preparation Containing Kojic Acid Using Hyperspectral Imaging—Preliminary Study. *Journal of Clinical Medicine,* 12**,** 2710.

WEI, X., LUO, D., YAN, Y., YU, H., SUN, L., WANG, C., SONG, F., GE, H., QIAN, H., LI, X., TANG, X. & LIU, P. 2019. Kojic acid inhibits senescence of human corneal endothelial cells via NF-κB and p21 signaling pathways. *Experimental Eye Research,* 180**,** 174-183. DOI: https://doi.org/10.1016/j.exer.2018.12.020.

WELSH, G. I., WILSON, C. & PROUD, C. G. 1996. GSK3: a SHAGGY frog story. *Trends in cell biology,* 6**,** 274-279.

WORTZEL, I. & SEGER, R. 2011. The ERK cascade: distinct functions within various subcellular organelles. *Genes & cancer,* 2**,** 195-209.

WU, L., YAN, X., SUN, R., MA, Y., YAO, W., GAO, B., ZHANG, Q., YOU, J., WANG, H., HAN, Q. & SUN, X. 2023. Sirt3 restricts tumor initiation via promoting LONP1 deacetylation and K63 ubiquitination. *Journal of Translational Medicine,* 21**,** 81. DOI: 10.1186/s12967-023-03925-x.

WU, Y., SHI, Y. G., ZENG, L. Y., PAN, Y., HUANG, X. Y., BIAN, L. Q., ZHU, Y. J., ZHANG, R. R. & ZHANG, J. 2019. Evaluation of antibacterial and anti-biofilm properties of kojic acid against five food-related bacteria and related subcellular mechanisms of bacterial inactivation. *Food Science and Technology International,* 25**,** 3-15. DOI: 10.1177/1082013218793075.

XIANG, M., SHI, X., LI, Y., XU, J., YIN, L., XIAO, G., SCOTT, M. J., BILLIAR, T. R., WILSON, M. A. & FAN, J. 2011. Hemorrhagic shock activation of NLRP3 inflammasome in lung endothelial cells. *The Journal of Immunology,* 187**,** 4809-4817.

XIAO, Y. Q., MALCOLM, K., WORTHEN, G. S., GARDAI, S., SCHIEMANN, W. P., FADOK, V. A., BRATTON, D. L. & HENSON, P. M. 2002. Cross-talk between ERK and p38 MAPK mediates selective suppression of pro-inflammatory cytokines by transforming growth factor-beta. *Journal of Biological Chemistry,* 277**,** 14884-93. DOI: 10.1074/jbc.M111718200.

XIONG, Y., WANG, L., WANG, S., WANG, M., ZHAO, J., ZHANG, Z., LI, X., JIA, L. & HAN, Y. 2018. SIRT3 deacetylates and promotes degradation of P53 in PTEN-defective non-small cell lung cancer. *Journal of Cancer Research and Clinical Oncology,* 144**,** 189-198. DOI: 10.1007/s00432-017-2537-9.

YABUTA, T. 1924. The constitution of kojic acid, a γ-pyrone derivative formed by Aspergillus Oryzæ from carbohydrates. *Journal of the Chemical Society, Transactions,* 125**,** 575-587. DOI: 10.1039/CT9242500575.

YAMAMOTO, H., SCHOONJANS, K. & AUWERX, J. 2007. Sirtuin Functions in Health and Disease. *Molecular Endocrinology,* 21**,** 1745-1755. DOI: 10.1210/me.2007-0079.

YANG, W., NAGASAWA, K., MÜNCH, C., XU, Y., SATTERSTROM, K., JEONG, S., HAYES, S. D., JEDRYCHOWSKI, M. P., VYAS, F. S. & ZAGANJOR, E. 2016. Mitochondrial sirtuin network reveals dynamic SIRT3-dependent deacetylation in response to membrane depolarization. *Cell,* 167**,** 985-1000. e21.

YAPRYNTSEVA, M. A., MAXIMCHIK, P. V., ZHIVOTOVSKY, B. & GOGVADZE, V. 2022. Mitochondrial sirtuin 3 and various cell death modalities. *Frontiers in Cell and Developmental Biology,* 10**,** 947357. DOI: 10.3389/fcell.2022.947357.

YUAN, Q., ZENG, Z. L., YANG, S., LI, A., ZU, X. & LIU, J. 2022. Mitochondrial Stress in Metabolic Inflammation: Modest Benefits and Full Losses. *Oxidative Medicine and Cellular Longevity,* 2022**,** 8803404. DOI: 10.1155/2022/8803404.

ZALEŚNA, I., HARTMAN, M. L. & CZYŻ, M. 2016. BRAF mutation in progression and therapy of melanoma, papillary thyroid carcinoma and colorectal adenocarcinoma. *Advances in Hygiene and Experimental Medicine,* 70**,** 471-488.

ZHANG, H. & SUN, S.-C. 2015. NF-κB in inflammation and renal diseases. *Cell & bioscience,* 5**,** 1-12.

ZHANG, J., CHEN, H., CHEN, M., WANG, H., WANG, Q., SONG, X., HAO, H. & FENG, Z. 2017. Kojic acid-mediated damage responses induce mycelial regeneration in the basidiomycete Hypsizygus marmoreus. *PLOS ONE,* 12**,** e0187351. DOI: 10.1371/journal.pone.0187351.

ZHANG, J., XIANG, H., LIU, J., CHEN, Y., HE, R. R. & LIU, B. 2020. Mitochondrial Sirtuin 3: New emerging biological function and therapeutic target. *Theranostics,* 10**,** 8315-8342. DOI: 10.7150/thno.45922.

ZHANG, X., FAN, C., ZHANG, H., ZHAO, Q., LIU, Y., XU, C., XIE, Q., WU, X., YU, X. & ZHANG, J. 2016. MLKL and FADD are critical for suppressing progressive lymphoproliferative disease and activating the NLRP3 inflammasome. *Cell reports,* 16**,** 3247-3259.

ZHONG, Z., UMEMURA, A., SANCHEZ-LOPEZ, E., LIANG, S., SHALAPOUR, S., WONG, J., HE, F., BOASSA, D., PERKINS, G. & ALI, S. R. 2016. NF-κB restricts inflammasome activation via elimination of damaged mitochondria. *Cell,* 164**,** 896-910.

ZHONG, Z., ZHAI, Y., LIANG, S., MORI, Y., HAN, R., SUTTERWALA, F. S. & QIAO, L. 2013. TRPM2 links oxidative stress to NLRP3 inflammasome activation. *Nature communications,* 4**,** 1611.

ZHOU, J.-Y., LIU, Y. & WU, G. S. 2006. The Role of Mitogen-Activated Protein Kinase Phosphatase-1 in Oxidative Damage–Induced Cell Death. *Cancer research,* 66**,** 4888-4894.

ZHOU, R., YAZDI, A. S., MENU, P. & TSCHOPP, J. 2011. A role for mitochondria in NLRP3 inflammasome activation. *Nature,* 469**,** 221-225.

ZILLES, J. C., DOS SANTOS, F. L., KULKAMP-GUERREIRO, I. C. & CONTRI, R. V. 2022. Biological activities and safety data of kojic acid and its derivatives: A review. *Experimental Dermatology,* 31**,** 1500-1521. DOI: https://doi.org/10.1111/exd.14662.

CHAPTER 2

Exploring the biochemical effects of kojic acid and its implications in humans

This chapter includes a review of literature on the biochemical effects of kojic acid in humans. This chapter highlights the key role of kojic acid in oxidative stress, inflammation, and cancer. Furthermore, the review focuses on gaps in research and the need for more studies in human models to determine the compounds toxicity.

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Exploring the biochemical effects of kojic acid and its implications in humans

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Abstract

Kojic acid is known to inhibit melanin production, a property widely exploited in the cosmetic industry. Although well-known as a skin lightener, kojic acid has diverse applications in the food, agricultural, pharmaceutical, and medical industries. Kojic acid has been shown to possess antimicrobial properties favourable to the food and agricultural industry. However, many treatment options may be under-reported in the pharmaceutical and medical industries. In different models, kojic acid displayed favourable results against inflammation, oxidative stress, and cancer. KA displayed instability and low efficacy in some treatment applications. KA derivatives developed to overcome these shortcomings provide innovative prospects in targeting biochemical pathways. This review assessed the advancement in kojic acid research in humans, evaluating common routes of exposure and the efficacy of its use. The body of literature reviewed showed low toxicity with many viable avenues for future research.

Keywords: Inflammation, Kojic acid, Oxidative stress, Tyrosinase Inhibition, Cancer

Introduction

According to the World Health Organization (WHO), skin lighteners are popular among Asian and African populations. Statistics reflect that 60% of Indians, 41% of Chinese, and 77% of Nigerians regularly use skin lighteners (Chib et al., 2023). The popularity of kojic acid (KA) is rising due to the cosmetic industry promoting its use. The market for skin-lightening creams is expected to grow significantly, with an estimated value of $31.2 billion by 2024 (Chib et al., 2023). KA is a product of the biosynthetic conversion of sugar to γ-pyrone in genera *Aspergillus* and *Penicillium* (Parrish et al., 1966, Mohamad et al., 2010, El-Kady et al., 2014, Rasmey and Abdel-Kareem, 2021, Phasha et al., 2022). Presently, KA's popularity has grown exponentially and is estimated to rise to be a $39 million industry (Felipe et al., 2023). With the increase in demand, production has been optimised to supply large quantities of KA (Chib et al., 2023, de Caldas Felipe et al., 2023).

Although first described by Saito (1907), the first comprehensive study describing the production of KA by *A. oryzae* was from the fermentation of steamed rice(Yabuta, 1924, El-Aasar, 2006, Ayuhastuti et al., 2024). Carbon sources can produce KA, including arabinose, acetate, ethanol, glucose, sucrose, and xylose (Burdock et al., 2001). KA can be chemically produced by acetylation/deacetylation reactions (Lassfolk et al., 2019).

KA has several uses in the agricultural, food, cosmetic, and medicinal industries (Figure 2.1). The broad span of uses can be attributed to its structural similarities with flavonoids, compounds found in many plants. KA has been used as a pesticide and insecticide due to its antifungal and antibacterial properties (Lee et al., 1950, Beard and Walton, 1969, Uher et al., 1994, Kim et al., 2012, Wu et al., 2019). KA is widely used to preserve food by preventing the discolouration of fruit and as a flavour-enhancing

additive (Chen et al., 1991, Burnett et al., 2010b, Deri et al., 2016). KA is a common ingredient in many Japanese traditional foods and beverages (Kotyzova et al., 2004). Consumption of KA was found to be safe, with no severe health concerns (Burdock et al., 2001). Studies have been carried out to determine the influence of Japanese diets on their longevity. It was found that the Japanese consume an array of fermented foods, largely *koji* (fermented rice), from *A.oryzae* or *A. luchuensis* (Machida et al., 2008). The study found that KA has a large amount of glycosylceramide, which is digested by the intestinal microbial flora and increases *Blautia coccoides* (Hamajima et al., 2016). Microbial flora has shown an effect on individuals' health and can play a key role in longevity and good health.

KA is primarily utilized in cosmetics. As a result, skin is particularly vulnerable to KA. The cosmetic industry's increased interest in KA was sparked due to its ability to inhibit tyrosinase activity (Bentley, 2006, Hasil et al., 2020, Suryadi et al., 2022). By assessing the compound's toxicity, we can establish if the compound is safe for use in cosmetic products. Medical applications include anti-inflammatory, antioxidant, and anti-cancer activity (Zilles et al., 2022, Suthiram et al., 2023). Many skin conditions are caused by the dysregulation of melanin production in humans. KA has shown the potential to treat skin disorders such as leishmaniasis by decreasing the parasitic load (Rodrigues et al., 2014, Brtko, 2022, Saeedi et al., 2023).

At discovery, KA was classified as a mycotoxin due to its contamination of maize and poultry feed and co-occurrence with aflatoxins (Parrish et al., 1966, Souza et al., 2013). With the emergence of new research into KA, some researchers suggest that KA does not pose a mycotoxin risk to human health (Bentley, 2006). The lack of research in some aspects of the efficacy of KA on human health may be the reason for KA's varying classification. This draws attention to the need for more research into the health effects of KA. This review aims to assess the biochemical effects of KA by evaluating common routes of exposure.

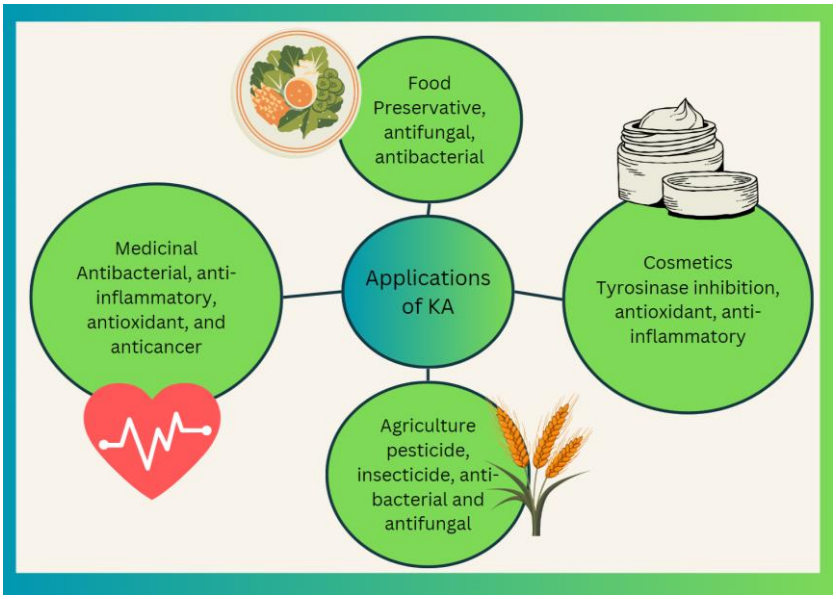

Figure 2.1: Applications that exploit kojic acid attributes

Chemical structure and properties

KA is known by the chemical nomenclature 5-hydroxy-2-hydroxymethyl-γ-pyranone ($C_6H_6O_4$). Saito (1907) first discovered KA, but Yabuta (1924) only later determined the structure. KA is a weak, heterocyclic acid with a hydroxymethyl group and a hydroxyl group in the C2 and C5 positions respectively (Yabuta, 1924, Beelik, 1956, Ichimoto et al., 1965). KA has a stable amylene oxide ring structure. The compound structure allows for the formation of salts by the chelation of metals such as sodium, copper, zinc, calcium, nickel, and cadmium (Beelik, 1956, Coupland and Niehaus, 1987, Saeedi et al., 2019). This attribute has been exploited in the inhibition of melanin. The formation of coloured iron complexes is irreversible (Friedemann, 1934). The compound is soluble in water, ethanol, or acetone (Burdock et al., 2001).

Absorption, distribution, metabolism, and excretion (ADME)

KA is absorbed and distributed in the body through the dermal, transdermal, and oral routes of exposure. An *in vitro* study on human dermatomed skin found that the percutaneous absorption of 2 mg/cm^3 KA was 16.98% of the administered KA following 16 h incubation. This study was further validated in humans. Women who applied creams containing 1% KA to their skin for periods ranging from 0.5-24 h were found to have 1 ng/ml KA in their plasma. This study found low penetration of KA into the bloodstream with no adverse effects being identified (Sansho Seiyaku Co., 2001).

The consumption of fermented food containing undetermined quantities of KA showed blood plasma concentrations of 1-8 µg/ml within 12-24 h. This, as well as other studies, indicated that KA was absorbed by the gastrointestinal tract (Niwa and Akamatsu, 1991, Higa et al., 2000). According to the International Agency for Research on Cancer (IARC), studies looking into the metabolism of KA have

not been established. KA showed unfavourable absorption, distribution, metabolism and excretion potential (Emami et al., 2022).

Regulations on kojic acid usage

The European Commission's SCCP determined a maximum concentration of 1% KA in skincare products due to health concerns reflected in published work on the effect of thyroid and skin sensitisation (SCCP, 2021). Furthermore, the Cosmetic Ingredient Review (CIR) concluded that concentrations below 1% cannot lighten skin and cause sensitisation (Chib et al., 2023). KA's use as a seafood preservative was found to have an approximate concentration of 0.25%-0.6% of KA. In terms of ingestion, KA is a fermentative by-product that is found in small quantities in food. Previous research has found the LD_{50} dose in mammals to be 1 g/kg (Brtko et al., 2004b). The absorption and distribution of KA were found to be negligible from dermal penetration. Therefore, the metabolism of KA will significantly lower the distributed concentration.

Kojic acid inhibits melanogenesis

Melanogenesis is controlled by several intrinsic factors such as hormones, genes and the immune system while extrinsic factors include ultraviolet and chemical exposure (Talebi et al., 2022). The primary role of melanin is to protect skin and eyes from damage. Melanogenesis is a multi-step process. Melanin is derived from tyrosine and is produced by melanocytes (Wawrzyk-Bochenek et al., 2023). Polyphenol oxidase (tyrosinase) is a glycoprotein found in melanosomes in the epidermis. The enzyme is responsible for the colour of epidermal cells in the skin, eyes, and hair of animals (Pekkarinen et al., 1999). Tyrosinase is a metalloprotein enzyme containing histidine residues that bind copper ions at its active site and is responsible for the biosynthesis of L-DOPA (Pawelek and Körner, 1982, Mayer, 1986, Cabanes et al., 1994, Gillbro and Olsson, 2011). Tyrosinase catalyses two steps in melanogenesis: (1) hydroxylation of tyrosine to L-DOPA as monophenolase and (2) oxidation of L-DOPA to dopaquinone (Kim et al., 2003) (Figure 2.2). The subsequent biosynthetic steps are non-enzymatic, thus making tyrosinase a key enzyme in the rate-limiting steps. The differing factor between monophenolase and diphenolase activity is determined by the hydroxyl molecule found between the copper ions (Baber et al., 2023).

KA chelating properties are crucial in the inhibition of tyrosinase activity. KA chelates transition metals such as ferrous (Fe^{3+}) and copper (Cu^{2+}). In cosmetics, KA inhibits the process by chelating the copper ion (II) found in tyrosinase hence decreasing melanin production (Chen et al., 2013). KA is a slow-binding inhibitor that binds to active tyrosinase (Chang, 2009). KA could be used to reduce wrinkles because it scavenges free radicals allowing it to serve as an anti-ageing agent (Saeedi et al., 2023).
KA is hydrophilic which limits the absorption of the compound in cosmetic applications. This attribute affects permeability, bioavailability and half-life in biological material (Saeedi et al., 2023). Due to

KA's inability to sufficiently penetrate the skin, solid lipid nanoparticles loaded with KA were developed. The result showed increased tyrosinase inhibition than that of KA alone (Khezri et al., 2020). Other preliminary studies showed that 3% KA was effective in reducing hyperpigmentation caused by acne (Wawrzyk-Bochenek et al., 2023). Similarly, KA derivatives containing amides. triazole analogues, and phenol, showed more potent inhibition of tyrosinase than KA alone and limited toxicity (Noh et al., 2007, Noh et al., 2009, Ashooriha et al., 2019, Ashooriha et al., 2020). KA possesses many other attributes that potentially aid in its anti-melanogenic effect (Moon et al., 2001, Ma et al., 2011, Wawrzyk-Bochenek et al., 2023).

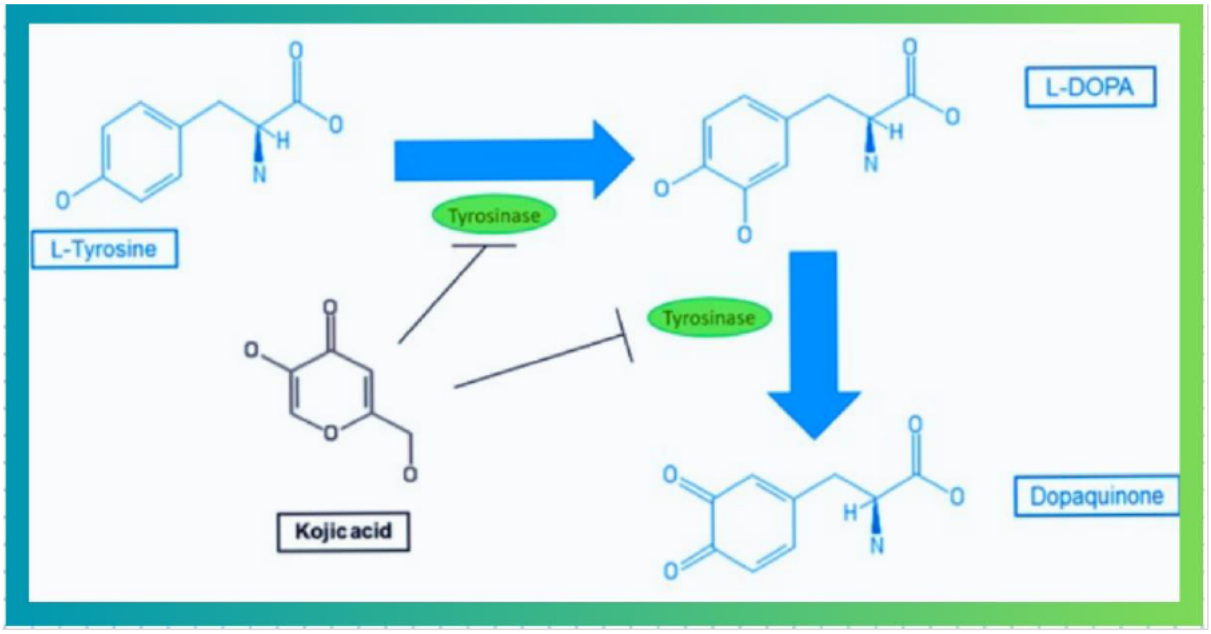

Figure 2.2: Overview of tyrosinase inhibition by kojic acid in melanin production. Chemical structures were drawn using PubChem Sketcher Version 2.4.

Other skin-lightening compounds such as hydroquinone are also tyrosinase inhibitors. Hydroquinone was a preferred treatment for hyperpigmentation; however, it has chronic side effects such as nephrotoxicity and cancer (Findlay et al., 1975, Kooyers and Westerhof, 2004). Due to safety concerns, hydroquinone is not used as a treatment for hyperpigmentation. In 2000, the Food and Drug Administration (FDA) banned the use of hydroquinone for skin lightening in favour of naturally produced alternatives such as KA (Kwon et al., 2016, Chib et al., 2023). KA has become a more popular option due to its potent inhibition of tyrosinase, often used as a positive control in research (Chang, 2009).

Additional effects of kojic acid

KA studies have investigated several pharmaceutical and medical properties to determine potential applications. KA is commonly referred to as an anticancer, antioxidant, and anti-inflammatory agent (Saeedi et al., 2019, Brtko, 2022).

In vitro toxicity studies

For the purposes of the review, a summary of *in vitro* studies was compiled to highlight the biochemical effects of kojic acid in different cell lines (Table 2.1). The *in vitro* studies illustrated KA's potential role in cancer, inflammation, and oxidative stress.

Table 2.1: Advances in the toxicity profile of KA in different in vitro models

	***In vitro* model**	**Biochemical effects**	**References**
Cancer	Epithelial cells	KA [8 µg/ml] (safe recommended concentration) was used to treat A375 cells for 24, 48, and 72 h. KA decreased proteins responsible for tumour growth and survival.	(Nawarak et al., 2008)
		KA-induced cytotoxic effects on B16F1 melanoma cells at higher concentrations [125- 500 µg/ml] but not at lower concentrations [7.81- 31.25 µg/ml] after a 72 h treatment.	(Lajis et al., 2012b)
	Cervical cells	HeLa cells, treated with KA (25 µg) for 48 h, did not induce cell cycle arrest in the absence and presence of 50 µM copper ion.	(Chen et al., 2013)
	Liver cells	KA [0- 12.67 mM] dose-dependently decreased HepG2 cell viability following a 24 h treatment.	(Suthiram et al., 2023)
Inflammation	Epithelial cells	Human transfected-HaCaT and SCC-13 cells treated with 10 mM KA for 24 h showed inhibition of NFκB activation.	(Moon et al., 2001)
		KA [100 µM] was found to have senescence inhibition potential through NFκB and p21 pathways in human corneal epithelial cells (HCEC) following treatment for 1, 3, 5, and 7 days	(Wei et al., 2019)
		KA [0.2 mM] inhibited melanogenesis by stimulating interleukin-6 (IL-6) in	(Choi et al., 2012)

		keratinocyte and melanocyte co-cultures following treatment for 3 days.	
	Immune cells	KA [50 µg/ml] were not cytotoxic to monocytes and increased IL-6 production.	(Da Costa et al., 2018)
	Liver cells	HepG2 cells treated with KA [8.02 mM] suppressed inflammatory marker, NFκB, following 24 h.	(Suthiram et al., 2023)
Oxidative stress	Immune cells	Neutrophils and lymphocytes showed reduced ROS following KA exposure [5 and 20 µg/ml] in both xanthine oxidase systems.	(Niwa and Akamatsu, 1991)
	Liver cells	KA [10.34 µg/ml] displayed potent free radical scavenging ability in HepG2 cells.	(El-Metwally et al., 2020)

***In vivo* toxicity studies**

To determine sensitivity, 107 patients with chloasma were treated with KA creams [2.5% KA] twice daily for an average of 9.5 months. Two individuals developed facial dermatitis due to hypersensitivity. No sensitivity was detected in 66 patients with chloasma (Nakayama, 1982) and 31 healthy subjects when treated with the cream [1% KA] (Hira et al., 1985). According to studies carried out by Nakayama (1982), no sensitivity was detected following the use of KA-containing creams. In a similar study, the frequency of sensitisation to KA was assessed in patch tests of 220 female patients with suspected cosmetic-related dermatitis. Of the total patients, 8 patients used one skin product with KA. Five reacted to one or more products containing 1% KA. Three remaining patients showed no reaction to products containing KA. The 212 patients who did not use KA previously showed no adverse results (Nakagawa et al., 1995). Another clinical trial showed the effectiveness of 1% KA in treating melasma and decreasing age spots and freckles (Mishima et al., 1994, Ando et al., 2010). There are scarce studies assessing the effect of solely KA treatment.

One trial used a serum combination of 3% tranexamic, 1% KA and 5% niacinamide to topically treat melasma and hyperpigmentation (Desai et al., 2019). The combination treatment proved effective in treating melasma and post-inflammatory hyperpigmentation. Another combination treatment containing 2% KA, 2% hydroquinone and 10% glycolic acid was used to treat 40 women with melasma over 12 weeks. Results showed a reduction in melasma in 24 women (60%), who received KA treatment. The side effects experienced by these women were redness, exfoliation, and a stinging sensation (Lim, 1999, Tetali et al., 2020).

Combination treatment of vitamin C and KA was compared to hydroquinone when treating patients with dyschromia. It was found that the combination of vitamin C and KA yielded a more favourable outcome than that of hydroquinone over 12 weeks. Patients had improved skin tone and dyschromia with less side effects (Oresajo et al., 2008). A study by Draelos et al. (2010) assessed the efficacy of a combination of glycolic acid, Emblica plant extract and KA to 4% hydroquinone treatment on patients with dyschromia. The 12-week trial, comprising of 80 patients, found a similar result for both treatments. Hence, this combination treatment was as effective as hydroquinone and is a viable alternate treatment (Draelos et al., 2010, Tetali et al., 2020). In another 39-patient clinical trial, KA combination therapy was used to treat hyperpigmentation. The patients were treated with 5% glycolic acid and 2% KA on one half of their face whilst the other half was treated with 5% glycolic acid and hydroquinone. The results revealed that 28% saw improvement on the KA side and 21% saw improvement on the hydroquinone side (Gupta et al., 2006).

Genotoxicity studies

KA [1250, 2500, 5000 µg/ml] was used to treat wild-type *p53* WTK1 and TK1 lymphoblast cells and showed DNA damage. KA induced DNA single-strand breaks (SSBs) (Kawaguchi et al., 2007). Thymidine kinase (TK) mutation assays revealed increased fractions of normal growth not mutant growth. This showed that point mutations at the *TK locus* occurred; however, structural changes that form chromosomal aberrations did not occur (Kawaguchi et al., 2007). Lastly, human keratinocytes (SVK14) treated with KA [1000, 2000, 4000 µg/ml] for 3 h with/without S9 and recovery for 48 h], KA [1000, 2000, 4000 µg/ml for 24 h without S9 and recovery for 48 h] and KA [1000, 2000, 4000 µg/ml for 24 h with/without S9 and recovery for 72 h] showed no micronuclei was induced in all exposures (Nohynek et al., 2004). KA did not exert significant genotoxic effects.

Research advancements in kojic acid derivatives

As mentioned previously, KA's diverse applications and characteristics are beneficial in many industries. In the medical industry, many treatments have harmful side effects, and resistance to antibiotics is becoming more prevalent. By advancing research into easily accessible compounds with favourable characteristics and minimal toxicity, treatment options for disease can become more effective. Drawbacks of KA such as instability and low permeability have been solved by the development of KA derivatives. Halogen, chloro- and bis-KA derivatives exerted anticancer effects in leukaemia cells (Bransová et al., 1995, Bransová et al., 1997, Peroković et al., 2020), cervical cancer cells (Hudecová et al., 1996, Peroković et al., 2020), breast adenocarcinoma cells (Fickova et al., 2008, Ercan et al., 2020), glioma cells (Yoo et al., 2010), melanoma cells (Karakaya et al., 2018), hepatocellular carcinoma cells (Oncul et al., 2019), colorectal adenocarcinoma cells, and

bronchioalveolar carcinoma cells (Peroković et al., 2020). Other favourable effects of derivatives include anti-inflammatory properties (Rho et al., 2010, Lee et al., 2019a).

Microbial effects of KA and KA-derivatives on bacteria, viruses, and fungi

KA is an antimicrobial that has displayed antibacterial, antiviral, and antifungal effects (Figure 2.3). KA esters aid in extending periods of drug release in the form of nano-emulsions. These esters are trapped close to the cell membrane enhancing passive cellular absorption and antimicrobial activity (Syed Azhar et al., 2020). The antibacterial activity of KA was illustrated in foodborne pathogens, namely *Listeria monocytogenes*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhimurium* (Liu et al., 2014b). It was found that the bacterial membrane integrity was altered as indicated by the leakage of intracellular enzymes and the release of potassium (K^+). The Gram-negative bacteria displayed a higher degree of damage to the cell membrane (Wu et al., 2019). The properties and structure suggest a possible use in drug discovery and treatments due to its low toxicity.

An increasing number of studies are exploiting KA as a pharmaceutical drug delivery system. Nanomedicine has become more prevalent in recent years to improve drug delivery and minimise side effects (Zhang et al., 2024). Research into KA and nanotechnology-based drug delivery systems used to treat ageing found favourable results with no cytotoxicity (Gonçalez et al., 2013). This shows the potential to increase efficacy in skin treatments. A study explored the potential medical applications of KA by using iron oxide magnetic nanoparticles loaded with KA. The study exploited KA's antimicrobial properties against Gram-negative and Gram-positive bacteria. The nanoparticles were used to optimize drug loading and decrease microbial burden (Hussein-Al-Ali et al., 2014). Another study showed that Halloysite nanotubes combined with KA displayed antibacterial activity against pathogens *E.coli, K. pneumoniae, S. aureus,* and *E. faecalis* (Patamia et al., 2023).

KA's role in the immune response of the body was further studied in macrophage activation. It was determined that KA could activate macrophages by cytoskeleton rearrangement, increasing cell surface exposure, ROS production, and the enhancement of phagocytic activity (Rodrigues et al., 2011). The activation of immune responses by KA suggests the potential for the treatment of pathogen-induced conditions.

Studies into KA and its derivatives found antiviral activity against RNA viruses (*Parainfluenza-3* virus) (Aytemir and Özçelik, 2010). This provides insight into potential treatment options for eradicating diseases caused by viruses that are often hard to treat. The fermentation of *Aspergillus* species results in the production of *koji*. *Koji* contains KA ranging between 0-9.5 mg/g (Kitagaki, 2021). The effect of KA on coronavirus disease 2019 (COVID-19) has not been elucidated however, KA could potentially

influence SARS-COV-2. Pyranonigrin A is found in *koji*, which can potentially be used to inhibit protease inhibitors in SARS-COV-2 (Miyaki et al., 2007, Rao et al., 2020).

Fungal infections in plants pose a risk to the agricultural and food industry affecting food quality and income. KA blocks the biosynthesis of melanin in soybean pathogens. KA in combination with carbendazim displayed a high protective ability against fungal pathogens. KA was highly stable in its function as an antifungal alone or in combination (Sun et al., 2022). However, human health can also be affected by fungal infections, especially skin infections. KA was found to have antifungal properties through its chemo-sensitising activity. KA disrupts fungi antioxidation systems and could be used as an antifungal agent in conjugation with other compounds to enhance its potency (Baláz et al., 1993, Kim et al., 2012).

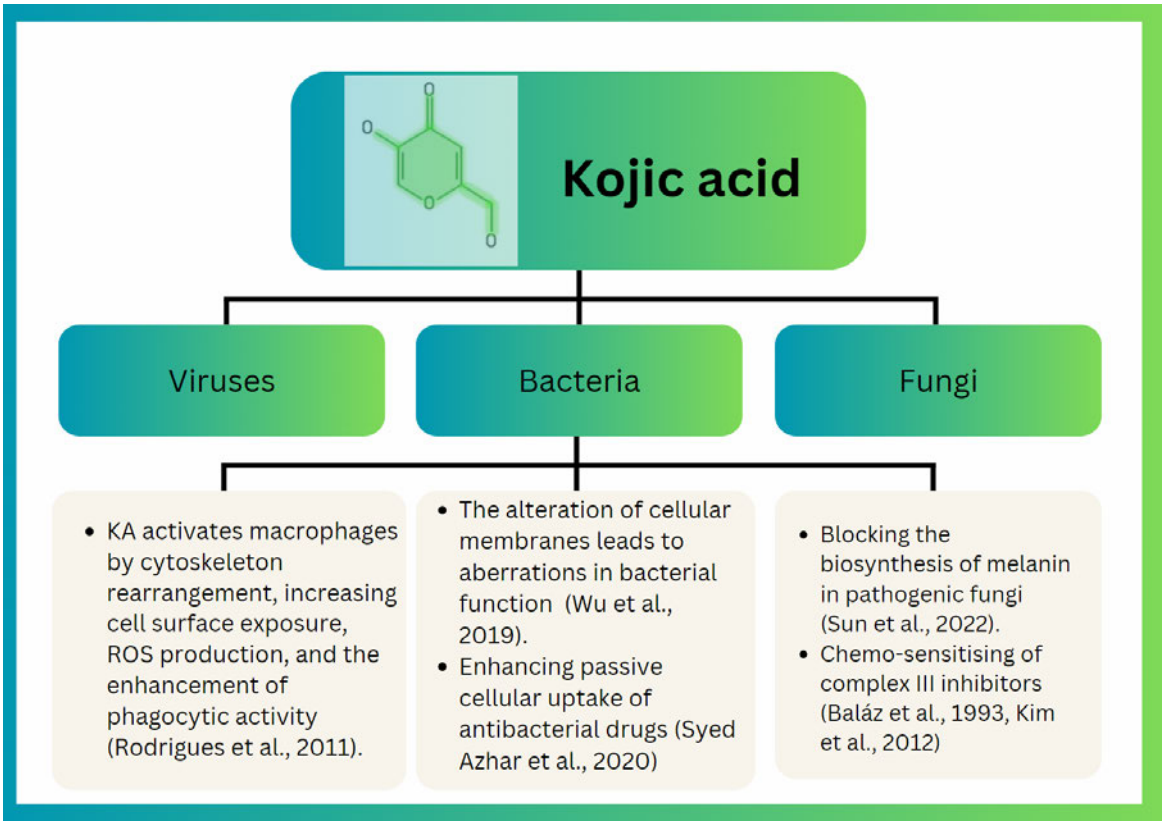

Figure 2.3: Summary of microbial applications of kojic acid.

There are still gaps in KA research in terms of the potential of the compound in the medicinal and pharmaceutical industries. KA has been shown to have antioxidant, antibacterial, anticancer, antifungal, and anti-inflammatory effects. Research assessing KA's capabilities in diseases is limited (Brtko et al., 2004b). A review of studies carried out showed low cytotoxicity. A drawback of KA monotherapy was the instability of the compound however, KA derivatives assessed are substantially more effective treatment options.

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Authors contributions

The review was conceptualised by the authors and represents an unbiased professional review of available literature. The conclusions drawn are solely those of the authors.

Competing interests

The authors declare no competing interests to declare that are relevant to the content of the article.

References

ANDO, H., MATSUI, M. S. & ICHIHASHI, M. 2010. Quasi-Drugs Developed in Japan for the Prevention or Treatment of Hyperpigmentary Disorders. *International Journal of Molecular Sciences,* 11**,** 2566-2575.

ASHOORIHA, M., KHOSHNEVISZADEH, M., KHOSHNEVISZADEH, M., MORADI, S. E., RAFIEI, A., KARDAN, M. & EMAMI, S. 2019. 1,2,3-Triazole-based kojic acid analogs as potent tyrosinase inhibitors: Design, synthesis and biological evaluation. *Bioorganic Chemistry,* 82**,** 414-422. DOI: 10.1016/j.bioorg.2018.10.069.

ASHOORIHA, M., KHOSHNEVISZADEH, M., KHOSHNEVISZADEH, M., RAFIEI, A., KARDAN, M., YAZDIAN-ROBATI, R. & EMAMI, S. 2020. Kojic acid-natural product conjugates as mushroom tyrosinase inhibitors. *European Journal of Medicinal Chemistry,* 201**,** 112480. DOI: 10.1016/j.ejmech.2020.112480.

AYUHASTUTI, A., SYAH, I. S. K., MEGANTARA, S. & CHAERUNISAA, A. Y. 2024. Nanotechnology-Enhanced Cosmetic Application of Kojic Acid Dipalmitate, a Kojic Acid Derivate with Improved Properties. *Cosmetics,* 11**,** 21.

AYTEMIR, M. D. & ÖZÇELIK, B. 2010. A study of cytotoxicity of novel chlorokojic acid derivatives with their antimicrobial and antiviral activities. *European Journal of Medicinal Chemistry,* 45**,** 4089-4095. DOI: https://doi.org/10.1016/j.ejmech.2010.05.069.

BABER, M. A., CRIST, C. M., DEVOLVE, N. L. & PATRONE, J. D. 2023. Tyrosinase Inhibitors: A Perspective. *Molecules,* 28**,** 5762.

BALÁZ, S., UHER, M., BRTKO, J., VEVERKA, M., BRANSOVÁ, J., DOBIAS, J., PÓDOVÁ, M. & BUCHVALD, J. 1993. Relationship between antifungal activity and hydrophobicity of kojic acid derivatives. *Folia microbiologica,* 38**,** 387-391. DOI: 10.1007/bf02898762.

BEARD, R. L. & WALTON, G. S. 1969. Kojic acid as an insecticidal mycotoxin. *Journal of Invertebrate Pathology,* 14**,** 53-59. DOI: https://doi.org/10.1016/0022-2011(69)90010-X.

BEELIK, A. 1956. Kojic acid. *Advances in Carbohydrate Chemistry and Biochemistry,* 48**,** 145-183.

BENTLEY, R. 2006. From miso, saké and shoyu to cosmetics: a century of science for kojic acid. *Natural Products Reports,* 23**,** 1046-1062. DOI: 10.1039/b603758p.

BRANSOVÁ, J., BRTKO, J., UHER, M. & NOVOTNÝ, L. 1995. Antileukemic activity of 4-pyranone derivatives. *International Journal of Biochemistry and Cell Biology,* 27**,** 701-6. DOI: 10.1016/1357-2725(95)00031-j.

BRANSOVÁ, J., UHER, M., NOVOTNÝ, L. & BRTKO, J. 1997. 5-benzyloxy-2-thiocyanatomethyl-4-pyranone, a novel heterocyclic compound: synthesis, structure determination and effects on neoplastic cell growth. *Anticancer Research,* 17**,** 1175-8.

BRTKO, J. 2022. Biological functions of kojic acid and its derivatives in medicine, cosmetics, and food industry: Insights into health aspects. *Archiv der Pharmazie,* 355**,** 2200215. DOI: https://doi.org/10.1002/ardp.202200215.

BRTKO, J., RONDAHL, L., FICKOVÁ, M., HUDECOVÁ, D., EYBL, V. & UHER, M. 2004. Kojic acid and its derivatives: history and present state of art. *Central European Journal of Public Health,* 12 Suppl**,** 16-18.

BURDOCK, G. A., SONI, M. G. & CARABIN, I. G. 2001. Evaluation of health aspects of kojic acid in food. *Regulatory Toxicology and Pharmacology,* 33**,** 80-101. DOI: 10.1006/rtph.2000.1442.

BURNETT, C. L., BERGFELD, W. F., BELSITO, D. V., HILL, R. A., KLAASSEN, C. D., LIEBLER, D. C., MARKS, J. G., SHANK, R. C., SLAGA, T. J., SNYDER, P. W. & ANDERSEN, F. A. 2010. Final Report of the Safety Assessment of Kojic Acid as Used in Cosmetics. *International Journal of Toxicology,* 29**,** 244-273. DOI: 10.1177/1091581810385956.

CABANES, J., CHAZARRA, S. & GARCIA-CARMONA, F. 1994. Kojic acid, a cosmetic skin whitening agent, is a slow-binding inhibitor of catecholase activity of tyrosinase. *Journal of Pharmacy and Pharmacology,* 46**,** 982-985. DOI: 10.1111/j.2042-7158.1994.tb03253.x.

CHANG, T.-S. 2009. An updated review of tyrosinase inhibitors. *International Journal of Molecular Sciences,* 10**,** 2440-2475. DOI: 10.3390/ijms10062440.

CHEN, J. S., WEI, C. I. & MARSHALL, M. R. 1991. Inhibition mechanism of kojic acid on polyphenol oxidase. *Journal of Agricultural and Food Chemistry,* 39**,** 1897-1901. DOI: 10.1021/jf00011a001.

CHEN, Y.-H., LU, P.-J., HULME, C. & SHAW, A. Y. 2013. Synthesis of kojic acid-derived copper-chelating apoptosis inducing agents. *Medicinal Chemistry Research,* 22**,** 995-1003. DOI: 10.1007/s00044-012-0094-y.

CHIB, S., JAMWAL, V. L., KUMAR, V., GANDHI, S. G. & SARAN, S. 2023. Fungal production of kojic acid and its industrial applications. *Applied Microbiology and Biotechnology,* 107**,** 2111-2130. DOI: 10.1007/s00253-023-12451-1.

CHOI, H., KIM, K., HAN, J., CHOI, H., JIN, S. H., LEE, E. K., SHIN, D. W., LEE, T. R., LEE, A.-Y. & NOH, M. 2012. Kojic acid-induced IL-6 production in human keratinocytes plays a role in

its anti-melanogenic activity in skin. *Journal of Dermatological Science,* 66**,** 207-215. DOI: https://doi.org/10.1016/j.jdermsci.2012.03.002.

COUPLAND, K. & NIEHAUS, W. G. 1987. Effect of nitrogen supply, Zn2+, and salt concentration on kojic acid and versicolorin biosynthesis by *Aspergillus parasiticus*. *Experimental Mycology,* 11**,** 206-213. DOI: https://doi.org/10.1016/0147-5975(87)90006-5.

DA COSTA, J. P., RODRIGUES, A. P. D., FARIAS, L. H. S., FRADE, P. C. R., DA SILVA, B. J. M., DO NASCIMENTO, J. L. M. & SILVA, E. O. 2018. Biological effects of kojic acid on human monocytes in vitro. *Biomedicine & Pharmacotherapy,* 101**,** 100-106.

DE CALDAS FELIPE, M. T., DO NASCIMENTO BARBOSA, R., BEZERRA, J. D. P. & DE SOUZA-MOTTA, C. M. 2023. Production of kojic acid by Aspergillus species: Trends and applications. *Fungal Biology Reviews,* 45**,** 100313.

DERI, B., KANTEEV, M., GOLDFEDER, M., LECINA, D., GUALLAR, V., ADIR, N. & FISHMAN, A. 2016. The unravelling of the complex pattern of tyrosinase inhibition. *Scientific reports,* 6**,** 1-10. DOI: 10.1038/srep34993.

DESAI, S., AYRES, E., BAK, H., GREEN, D. & ZHENG, Q. 2019. Effect of a tranexamic acid, kojic acid, and niacinamide containing serum on facial dyschromia: a clinical evaluation. *Journal of Drugs in Dermatology,* 18**,** 454-459.

DRAELOS, Z. D., YATSKAYER, M., BHUSHAN, P., PILLAI, S. & ORESAJO, C. 2010. Evaluation of a kojic acid, emblica extract, and glycolic acid formulation compared with hydroquinone 4% for skin lightening. *Cutis,* 86**,** 153-8.

EL-AASAR, S. 2006. Cultural conditions studies on kojic acid production by Aspergillus parasiticus. *International Journal of Agriculture and Biology,* 8**,** 468-473.

EL-KADY, I. A., ZOHRI, A. N. A. & HAMED, S. R. 2014. Kojic acid production from agro-industrial by-products using fungi. *Biotechnology Research International,* 2014**,** 1-10.

EL-METWALLY, M. M., ELBEALY, E. R., BELTAGY, D. M., SHAABAN, M. & EL-KOTT, A. F. 2020. Suppressive efficiency of Kojic acid from Aspergillus tamarii MM11 against HepG-2 cell line derived from human liver cancer. *Tropical Journal of Pharmaceutical Research,* 19**,** 1661-1668.

EMAMI, S., AHMADI, R., AHADI, H. & ASHOORIHA, M. 2022. Diverse therapeutic potential of 3-hydroxy-4-pyranones and related compounds as kojic acid analogs. *Medicinal Chemistry Research,* 31**,** 1842-1861. DOI: 10.1007/s00044-022-02954-3.

ERCAN, A., ONCUL, S., KARAKAYA, G. & AYTEMIR, M. 2020. An allomaltol derivative triggers distinct death pathways in luminal a and triple-negative breast cancer subtypes. *Bioorganic Chemistry,* 105**,** 104403. DOI: 10.1016/j.bioorg.2020.104403.

FELIPE, M. T. D. C., BARBOSA, R. D. N., BEZERRA, J. D. P. & SOUZA-MOTTA, C. M. D. 2023. Production of kojic acid by Aspergillus species: Trends and applications. *Fungal Biology Reviews,* 45**,** 100313. DOI: https://doi.org/10.1016/j.fbr.2023.100313.

FICKOVA, M., PRAVDOVA, E., RONDHAL, L., UHER, M. & BRTKO, J. 2008. In vitro antiproliferative and cytotoxic activities of novel kojic acid derivatives: 5-benzyloxy-2-selenocyanatomethyl- and 5-methoxy-2-selenocyanatomethyl-4-pyranone. *Journal of Applied Toxicology,* 28**,** 554-9. DOI: 10.1002/jat.1300.

FINDLAY, G. H., MORRISON, J. G. & SIMSON, I. W. 1975. Exogenous ochronosis and pigmented colloid milium from hydroquinone bleaching creams. *British Journal of Dermatology,* 93**,** 613-622. DOI: 10.1111/j.1365-2133.1975.tb05110.x.

FRIEDEMANN, T. E. 1934. Chemical and Physiological properties of Kojic acid. *Science,* 80**,** 34. DOI: 10.1126/science.80.2063.34.

GILLBRO, J. M. & OLSSON, M. J. 2011. The melanogenesis and mechanisms of skin-lightening agents – existing and new approaches. *International Journal of Cosmetic Science,* 33**,** 210-221. DOI: 10.1111/j.1468-2494.2010.00616.x.

GONÇALEZ, M. L., CORRÊA, M. A. & CHORILLI, M. 2013. Skin Delivery of Kojic Acid-Loaded Nanotechnology-Based Drug Delivery Systems for the Treatment of Skin Aging. *BioMed Research International,* 2013**,** 271276. DOI: 10.1155/2013/271276.

GUPTA, A. K., GOVER, M. D., NOURI, K. & TAYLOR, S. 2006. The treatment of melasma: a review of clinical trials. *Journal of the American Academy of Dermatology,* 55**,** 1048-65. DOI: 10.1016/j.jaad.2006.02.009.

HAMAJIMA, H., MATSUNAGA, H., FUJIKAWA, A., SATO, T., MITSUTAKE, S., YANAGITA, T., NAGAO, K., NAKAYAMA, J. & KITAGAKI, H. 2016. Japanese traditional dietary fungus koji Aspergillus oryzae functions as a prebiotic for Blautia coccoides through glycosylceramide: Japanese dietary fungus koji is a new prebiotic. *Springerplus,* 5**,** 1-10. DOI: 10.1186/s40064-016-2950-6.

HASIL, A., MEHMOOD, A., NOUREEN, S. & AHMED, M. 2020. Experimental and theoretical charge density analysis of skin whitening agent kojic acid. *Journal of Molecular Structure,* 1216**,** 128295.

HIGA, Y., OHKUBO, A., KITAJIMA, S., HATORI, A. & KARIYA, K. 2000. Studies on thyroid function in rats subjected to repeated oral administration with Kojic acid *The Journal of Toxicological Sciences,* 25**,** 167-175. DOI: 10.2131/jts.25.3_167.

HIRA, Y., OHYAMA, Y., KAIMI, K., MISHIMA, Y. & FURUKAWA, T. 1985. Inhibitory effect of kojic acid contained cream in UV-induced pigmentationin human skin. *Japanese Cosmetic Science Society,* 9**,** 109.

HUDECOVÁ, D., JANTOVÁ, S., MELNÍK, M. & UHER, M. 1996. New azidometalkojates and their biological activity. *Folia Microbiologica (Praha),* 41**,** 473-6. DOI: 10.1007/bf02814660.

HUSSEIN-AL-ALI, S. H., EL ZOWALATY, M. E., HUSSEIN, M. Z., ISMAIL, M., DORNIANI, D. & WEBSTER, T. J. 2014. Novel kojic acid-polymer-based magnetic nanocomposites for

medical applications. *International Journal of Nanomedicine,* 9**,** 351-362. DOI: 10.2147/ijn.S53847.

IARC Kojic Acid. *International Agency into Research on Cancer Monographs,* 79**,** 607-618.

ICHIMOTO, I., FUJII, K. & TATSUMI, C. 1965. Studies on Kojic Acid and its Related γ-Pyrone Compounds. *Agricultural and Biological Chemistry,* 29**,** 325-330. DOI: 10.1080/00021369.1965.10858386.

KARAKAYA, G., ERCAN, A., ONCUL, S. & AYTEMIR, M. D. 2018. Synthesis and Cytotoxic Evaluation of Kojic Acid Derivatives with Inhibitory Activity on Melanogenesis in Human Melanoma Cells. *Anticancer Agents in Medicinal Chemistry,* 18**,** 2137-2148. DOI: 10.2174/1871520618666180402141714.

KAWAGUCHI, S., NAKAMURA, T., HONDA, G. & SASAKI, Y. F. 2007. Evaluation of in vitro Genotoxic Potential of Kojic Acid in Human Lymphoblastoid Cells. *Genes and Environment,* 29**,** 153-158. DOI: 10.3123/jemsge.29.153.

KHEZRI, K., SAEEDI, M., MORTEZA-SEMNANI, K., AKBARI, J. & ROSTAMKALAEI, S. S. 2020. An emerging technology in lipid research for targeting hydrophilic drugs to the skin in the treatment of hyperpigmentation disorders: kojic acid-solid lipid nanoparticles. *Artificial Cells, Nanomedicine, and Biotechnology,* 48**,** 841-853. DOI: 10.1080/21691401.2020.1770271.

KIM, D. H., HWANG, J. S., BAEK, H. S., KIM, K. J., LEE, B. G., CHANG, I., KANG, H. H. & LEE, O. S. 2003. Development of 5-[(3-aminopropyl)phosphinooxy]-2-(hydroxymethyl)-4H-pyran-4-one as a novel whitening agent. *Chemical and Pharmaceutical Bulletin* 51**,** 113-116. DOI: 10.1248/cpb.51.113.

KIM, J. H., CHANG, P.-K., CHAN, K. L., FARIA, N. C. G., MAHONEY, N., KIM, Y. K., MARTINS, M. D. L. & CAMPBELL, B. C. 2012. Enhancement of commercial antifungal agents by Kojic Acid. *International Journal of Molecular Sciences,* 13**,** 13867-13880. DOI: 10.3390/ijms131113867.

KITAGAKI, H. 2021. Medical Application of Substances Derived from Non-Pathogenic Fungi Aspergillus oryzae and A. luchuensis-Containing Koji. *Journal of Fungi,* 7**,** 243.

KOOYERS, T. J. & WESTERHOF, W. 2004. Toxicological aspects and health risks associated with hydroquinone in skin bleaching formula. *Nederlands Tijdschrift voor Geneeskunde,* 148**,** 768-771.

KOTYZOVA, D., EYBL, V., KOUTENSKY, J., BRTKO, J. & GLATTRE, E. 2004. Effects of kojic acid on oxidative damage and on iron and trace element level in iron-overloaded mice and rats. *Central European Journal of Public Health,* 12 Suppl**,** 41-44.

KWON, S.-H., HWANG, Y.-J., LEE, S.-K. & PARK, K.-C. 2016. Heterogeneous pathology of melasma and its clinical implications. *International journal of molecular sciences,* 17**,** 824.

LAJIS, A. F. B., HAMID, M. & ARIFF, A. B. 2012. Depigmenting Effect of Kojic Acid Esters in Hyperpigmented B16F1 Melanoma Cells. *Journal of Biomedicine and Biotechnology,* 2012**,** 952452. DOI: 10.1155/2012/952452.

LASSFOLK, R., SUONPÄÄ, A., BIRIKH, K. & LEINO, R. 2019. Chemo-enzymatic three-step conversion of glucose to kojic acid. *Chemical Communications,* 55**,** 14737-14740.

LEE, H. F., BOLTJES, B. & EISENMAN, W. 1950. Kojic Acid as an Inhibitor of Tubercle Bacilli. *American Review of Tuberculosis and Pulmonary Diseases,* 61**,** 738-741.

LEE, M., RHO, H. S. & CHOI, K. 2019. Anti-inflammatory Effects of a P-coumaric Acid and Kojic Acid Derivative in LPS-stimulated RAW264.7 Macrophage Cells. *Biotechnology and Bioprocess Engineering,* 24**,** 653-657. DOI: 10.1007/s12257-018-0492-1.

LIM, J. T. 1999. Treatment of melasma using kojic acid in a gel containing hydroquinone and glycolic acid. *Dermatologic Surgery,* 25**,** 282-4. DOI: 10.1046/j.1524-4725.1999.08236.x.

LIU, X., XIA, W., JIANG, Q., XU, Y. & YU, P. 2014. Synthesis, Characterization, and Antimicrobial Activity of Kojic Acid Grafted Chitosan Oligosaccharide. *Journal of Agricultural and Food Chemistry,* 62**,** 297-303. DOI: 10.1021/jf404026f.

MA, D.-L., XU, T., CHAN, D. S.-H., MAN, B. Y.-W., FONG, W.-F. & LEUNG, C.-H. 2011. A highly selective, label-free, homogenous luminescent switch-on probe for the detection of nanomolar transcription factor NF-kappaB. *Nucleic acids research,* 39**,** e67-e67.

MACHIDA, M., YAMADA, O. & GOMI, K. 2008. Genomics of Aspergillus oryzae: learning from the history of Koji mold and exploration of its future. *DNA Research,* 15**,** 173-183. DOI: 10.1093/dnares/dsn020.

MAYER, A. M. 1986. Polyphenol oxidases in plants-recent progress. *Phytochemistry,* 26**,** 11-20. DOI: https://doi.org/10.1016/S0031-9422(00)81472-7.

MISHIMA, Y., SHIBATA, T., SETO, H., OHYAMA, Y. & HATAE, S. 1994. Inhibitory action of kojic acid on melanogensis and its therapeutic effect for various human hyper-pigmentation disorders. *Skin research,* 36**,** 134-150.

MIYAKI, Y., ITO, C., ITOIGAWA, M. & OSAWA, T. 2007. Isolation of the Antioxidant Pyranonigrin-A from Rice Mold Starters Used in the Manufacturing Process of Fermented Foods. *Bioscience, Biotechnology, and Biochemistry,* 71**,** 2515-2521. DOI: 10.1271/bbb.70310.

MOHAMAD, R., MOHAMED, M., SUHAILI, N., SALLEH, M. & ARIFF, A. 2010. Kojic acid: Applications and development of fermentation process for production. *Biotechnology and Molecular Biology Reviews,* 5**,** 24-37.

MOON, K. Y., AHN, K. S., LEE, J. & KIM, Y. S. 2001. Kojic acid, a potential inhibitor of NF-kappaB activation in transfectant human HaCaT and SCC-13 cells. *Archives of Pharmacal Research,* 24**,** 307-311. DOI: 10.1007/bf02975097.

NAKAGAWA, M., KAWAI, K. & KAWAI, K. 1995. Contact allergy to kojic acid in skin care products. *Contact Dermatitis,* 32**,** 9-13. DOI: 10.1111/j.1600-0536.1995.tb00832.x.

NAKAYAMA, H. 1982. Treatment of chloasma with topical Kojic Acid. *Japanese Journal of Clinical Dermatology,* 36**,** 715-722.

NAWARAK, J., HUANG-LIU, R., KAO, S.-H., LIAO, H.-H., SINCHAIKUL, S., CHEN, S.-T. & CHENG, S.-L. 2008. Proteomics Analysis of Kojic Acid Treated A375 Human Malignant Melanoma Cells. *Journal of Proteome Research,* 7**,** 3737-3746. DOI: 10.1021/pr7008737.

NIWA, Y. & AKAMATSU, H. 1991. Kojic acid scavenges free radicals while potentiating leukocyte functions including free radical generation. *Inflammation,* 15**,** 303-315. DOI: 10.1007/BF00917315.

NOH, J.-M., KWAK, S.-Y., SEO, H., SEO, J.-H., KIM, B.-G. & LEE, Y.-S. 2009. Kojic acid-amino acid conjugates as tyrosinase inhibitors. *Bioorganic & medicinal chemistry letters,* 19**,** 5586-9. DOI: 10.1016/j.bmcl.2009.08.041.

NOH, J. M., KWAK, S. Y., KIM, D. H. & LEE, Y. S. 2007. Kojic acid-tripeptide amide as a new tyrosinase inhibitor. *Biopolymers,* 88**,** 300-7. DOI: 10.1002/bip.20670.

NOHYNEK, G. J., KIRKLAND, D., MARZIN, D., TOUTAIN, H., LECLERC-RIBAUD, C. & JINNAI, H. 2004. An assessment of the genotoxicity and human health risk of topical use of kojic acid [5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one]. *Food and Chemical Toxicology,* 42**,** 93-105. DOI: 10.1016/j.fct.2003.08.008.

ONCUL, S., KARAKAYA, G., DILSIZ AYTEMIR, M. & ERCAN, A. 2019. A kojic acid derivative promotes intrinsic apoptotic pathway of hepatocellular carcinoma cells without incurring drug resistance. *Chemical Biology and Drug Design***,** 1-10. DOI: 10.1111/cbdd.13615.

ORESAJO, C., YATSKAYER, M., FOLTIS, P. & HANSENNE, I. 2008. Comparative clinical assessment of two skin lightening products in female subjects with dyschromia. *Journal of the American Academy of Dermatology,* 58**,** AB26-AB26.

PARRISH, F. W., WILEY, B. J., SIMMONS, E. G. & LONG, L., JR. 1966. Production of aflatoxins and kojic acid by species of Aspergillus and Penicillium. *Applied microbiology,* 14**,** 139.

PATAMIA, V., ZAGNI, C., FIORENZA, R., FUOCHI, V., DATTILO, S., RICCOBENE, P. M., FURNERI, P. M., FLORESTA, G. & RESCIFINA, A. 2023. Total Bio-Based Material for Drug Delivery and Iron Chelation to Fight Cancer through Antimicrobial Activity. *Nanomaterials,* 13**,** 2036.

PAWELEK, J. M. & KÖRNER, A. M. 1982. The biosynthesis of mammalian melanin. *American Science,* 70**,** 136-145.

PEKKARINEN, S. S., HEINONEN, I. M. & HOPIA, A. I. 1999. Flavonoids quercetin, myricetin, kaemferol and (+)-catechin as antioxidants in methyl linoleate. *Journal of the Science of Food and Agriculture,* 79**,** 499-506. DOI: 10.1002/(sici)1097-0010(19990315)79:4<499::Aid-jsfa204>3.0.Co;2-u.

PEROKOVIĆ, V. P., CAR, Ž., USENIK, A., OPAČAK-BERNARDI, T., JURIĆ, A. & TOMIĆ, S. 2020. Adamantyl pyran-4-one derivatives and their in vitro antiproliferative activity. *Molecular Diversity,* 24**,** 253-263. DOI: 10.1007/s11030-019-09948-1.

PHASHA, V., SENABE, J., NDZOTOYI, P., OKOLE, B., FOUCHE, G. & CHUTURGOON, A. 2022. Review on the Use of Kojic Acid—A Skin-Lightening Ingredient. *Cosmetics,* 9**,** 64.

RAO, P., SHUKLA, A., PARMAR, P., RAWAL, R. M., PATEL, B., SARAF, M. & GOSWAMI, D. 2020. Reckoning a fungal metabolite, Pyranonigrin A as a potential Main protease (Mpro) inhibitor of novel SARS-CoV-2 virus identified using docking and molecular dynamics simulation. *Biophysical Chemistry,* 264**,** 106425.

RASMEY, A.-H. M. & ABDEL-KAREEM, M. 2021. Optimization of culture conditions for Kojic acid production in surface fermentation by Aspergillus oryzae isolated from wheat grains. *Bulletin of Pharmaceutical Sciences. Assiut,* 44**,** 201-211.

RHO, H. S., AHN, S. M., YOO, D. S., KIM, M. K., CHO, D. H. & CHO, J. Y. 2010. Kojyl thioether derivatives having both tyrosinase inhibitory and anti-inflammatory properties. *Bioorganic and Medicinal Chemistry Letters,* 20**,** 6569-71. DOI: 10.1016/j.bmcl.2010.09.042.

RODRIGUES, A. P., CARVALHO, A. S., SANTOS, A. S., ALVES, C. N., DO NASCIMENTO, J. L. & SILVA, E. O. 2011. Kojic acid, a secondary metabolite from Aspergillus sp., acts as an inducer of macrophage activation. *Cell Biology International,* 35**,** 335-343. DOI: 10.1042/cbi20100083.

RODRIGUES, A. P., FARIAS, L. H., CARVALHO, A. S., SANTOS, A. S., DO NASCIMENTO, J. L. & SILVA, E. O. 2014. A novel function for kojic acid, a secondary metabolite from Aspergillus fungi, as antileishmanial agent. *PLoS One,* 9**,** e91259. DOI: 10.1371/journal.pone.0091259.

SAEEDI, M., ESLAMIFAR, M. & KHEZRI, K. 2019. Kojic acid applications in cosmetic and pharmaceutical preparations. *Biomedicine and Pharmacotherapy,* 110**,** 582-593. DOI: 10.1016/j.biopha.2018.12.006.

SAEEDI, M., MORTEZA-SEMNANI, K., AKBARI, J., RAHIMNIA, S. M., AHMADI, F., CHOUBDARI, H., LOTFI, A. & HASHEMI, S. M. H. 2023. Development of kojic acid loaded collagen-chitosan nanoparticle as skin lightener product: In vitro and in vivo assessment. *Journal of Biomaterials Science, Polymer Edition***,** 1-17.

SAITO, K. 1907. Über die Säurebildung bei Aspergillus Oryzœ Vorläufige Mittheilung [On the formation of acid in Aspergillus Oryzae - Preliminary communication.]. *Shokubutsugaku Zasshi,* 21**,** 7-11.

SANSHO SEIYAKU CO., L. 2001. Absorption, distribution, metabolism, and excretion (ADME) of kojic acid in the rat. *Unpublished*.

SCCP 2021. Opinion on Kojic acid. *European Commission***,** 1-85.

SOUZA, M. D. L. M. D., SULYOK, M., FREITAS-SILVA, O., COSTA, S. S., BRABET, C., MACHINSKI JUNIOR, M., SEKIYAMA, B. L., VARGAS, E. A., KRSKA, R. &

SCHUHMACHER, R. 2013. Cooccurrence of Mycotoxins in Maize and Poultry Feeds from Brazil by Liquid Chromatography/Tandem Mass Spectrometry. *The Scientific World Journal,* 2013**,** 427369. DOI: 10.1155/2013/427369.

SUN, Q., ZHANG, S.-L., XIE, Y.-J., XU, M.-T., HERRERA-BALANDRANO, D. D., CHEN, X., WANG, S.-Y., SHI, X.-C. & LABORDA, P. 2022. Identification of New Fusarium sulawense Strains Causing Soybean Pod Blight in China and Their Control Using Carbendazim, Dipicolinic Acid and Kojic Acid. *International Journal of Environmental Research and Public Health,* 19**,** 10531.

SURYADI, H., IRIANTI, M. I. & SEPTIARINI, T. H. 2022. Methods of random mutagenesis of Aspergillus strain for increasing kojic acid production. *Current Pharmaceutical Biotechnology,* 23**,** 486-494.

SUTHIRAM, K. T., GHAZI, T., ABDUL, N. S. & CHUTURGOON, A. A. 2023. Kojic acid induces oxidative stress and anti-inflammatory effects in human hepatocellular carcinoma (HepG2) cells. *Toxicon,* 232**,** 107221. DOI: https://doi.org/10.1016/j.toxicon.2023.107221.

SYED AZHAR, S. N. A., ASHARI, S. E., AHMAD, S. & SALIM, N. 2020. In vitro kinetic release study, antimicrobial activity and in vivo toxicity profile of a kojic acid ester-based nanoemulsion for topical application. *Royal Society of Chemistry Advances,* 10**,** 43894-43903. DOI: 10.1039/d0ra04807k.

TALEBI, M., MAJIDI, K., BASSAM, K., ABDI, M., DANESHVAR, M., MOAYEDI, S. S., POURHESABI, S., ATTARROSHAN, M., BOUMI, S., KABIRI, M., HOSSEINI, F. S., KHOSHNEVISZADEH, M. & AMANLOU, M. 2022. Synthesis, biological evaluation, and molecular docking analysis of novel 1, 3, 4-thiadiazole -based kojic acid derivatives as tyrosinase inhibitors. *Journal of Molecular Structure,* 1268**,** 133707. DOI: https://doi.org/10.1016/j.molstruc.2022.133707.

TETALI, B., FAHS, F. M. & MEHREGAN, D. 2020. Popular over-the-counter cosmeceutical ingredients and their clinical efficacy. *International Journal of Dermatology,* 59**,** 393-405. DOI: https://doi.org/10.1111/ijd.14718.

UHER, M., KONECNY, V. & RAJNIAKOVA, O. 1994. Synthesis of 5-hydroxy-2-hydroxymethyl-4H-pyran-4-one derivatives with pesticide activity. *Chemical Papers,* 48**,** 282-284.

WAWRZYK-BOCHENEK, I., RAHNAMA, M., STACHURA, M., WILCZYŃSKI, S. & WAWRZYK, A. 2023. Evaluation of the Reduction of Skin Hyperpigmentation Changes under the Influence of a Preparation Containing Kojic Acid Using Hyperspectral Imaging—Preliminary Study. *Journal of Clinical Medicine,* 12**,** 2710.

WEI, X., LUO, D., YAN, Y., YU, H., SUN, L., WANG, C., SONG, F., GE, H., QIAN, H., LI, X., TANG, X. & LIU, P. 2019. Kojic acid inhibits senescence of human corneal endothelial cells via NF-κB and p21 signaling pathways. *Experimental Eye Research,* 180**,** 174-183. DOI: https://doi.org/10.1016/j.exer.2018.12.020.

WU, Y., SHI, Y. G., ZENG, L. Y., PAN, Y., HUANG, X. Y., BIAN, L. Q., ZHU, Y. J., ZHANG, R. R. & ZHANG, J. 2019. Evaluation of antibacterial and anti-biofilm properties of kojic acid against five food-related bacteria and related subcellular mechanisms of bacterial inactivation. *Food Science and Technology International,* 25**,** 3-15. DOI: 10.1177/1082013218793075.

YABUTA, T. 1924. The constitution of kojic acid, a γ-pyrone derivative formed by Aspergillus Oryzæ from carbohydrates. *Journal of the Chemical Society, Transactions,* 125**,** 575-587. DOI: 10.1039/CT9242500575.

YOO, D. S., LEE, J., CHOI, S. S., RHO, H. S., CHO, D. H., SHIN, W. C. & CHO, J. Y. 2010. A modulatory effect of novel kojic acid derivatives on cancer cell proliferation and macrophage activation. *Pharmazie,* 65**,** 261-6.

ZHANG, A., FAN, X., ZENG, X., XU, J., ZHOU, C., XIA, Q., SUN, Y., WU, Z. & PAN, D. 2024. Enhancing physicochemical, antimicrobial, and release properties of fish skin gelatin films using dual-layer nanoparticles loaded with tea polyphenols/kojic acid for air-dried chicken preservation. *Food Hydrocolloids,* 149**,** 109580. DOI: https://doi.org/10.1016/j.foodhyd.2023.109580.

ZILLES, J. C., DOS SANTOS, F. L., KULKAMP-GUERREIRO, I. C. & CONTRI, R. V. 2022. Biological activities and safety data of kojic acid and its derivatives: A review. *Experimental Dermatology,* 31**,** 1500-1521. DOI: https://doi.org/10.1111/exd.14662.

CHAPTER 3

Kojic acid induces oxidative, protein and mitochondrial stress in human melanoma (SK-MEL-1) cells

Kojic acid's chelating ability led to an evaluation of the compound's potential mitochondrial toxicity. KA chemo-sensitizes complex III inhibitors in the electron transport chain in fungi. This chapter investigates the mitotoxic potential of KA in human melanoma (SK-MEL-1) cells. The study explores the effect of KA on specific proteins involved in mitochondrial homeostasis and its implication on macromolecules and antioxidant response in SK-MEL-1 cells.

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Kojic acid induces oxidative, protein and mitochondrial stress in human melanoma (SK-MEL-1) cells

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Abstract

Kojic acid (KA, 5-hydroxy-2-hydroxymethyl-4-pyrone), a common skin lightener, chemo-sensitizes complex III inhibitors disrupting mitochondrial respiration in fungi; however, the mitochondrial effects of KA in humans have not been studied. This study determined the effect of KA on mitochondria in melanoma cells by assessing mitochondrial output (MTT assay and ATP luminometry), oxidative damage (protein carbonyls and TBARS), antioxidant responses and mitochondrial homeostasis proteins (Western Blot). KA increased cell viability and at 100 µg/ml displayed the greatest decrease in ATP levels. We assessed oxidative damage and the subsequent antioxidant responses to determine the reason for ATP depletion by KA. The oxidative stress marker (MDA) was increased at lower concentrations of KA (25 and 100 µg/ml); further, the antioxidant responses were initiated to reduce damage to susceptible macromolecules. KA-induced protein oxidation as evidenced by increased protein carbonyls and LON protease expression. Sirtuin 3 expression was decreased at all concentrations. In conclusion, KA caused lipid peroxidation and protein damage resulting in mitochondrial stress and initiated mitochondrial homeostasis.

Keywords: Kojic acid, mitochondrial stress, Nrf-2, Sirtuin 3, LON protease, protein carbonyls

Introduction

Kojic acid (KA, 5-hydroxy-2-hydroxymethyl-4-pyrone) is a fungal metabolite produced by the genera *Aspergillus* and *Penicillium* (Burdock et al., 2001, Ermis et al., 2023). KA has diverse uses ranging from skincare to food preservation. One percent of KA is permitted in cosmetic products due to health concerns including skin sensitisation and hyperplasia in the thyroid (Nakagawa et al., 1995, Burnett et al., 2010b, SCCP, 2021).

Several attributes of KA have been established by assessing the compound's biochemical effects in different models. KA has been categorised as an antibacterial and antifungal agent (Lee et al., 1950, Kim et al., 2012, Wu et al., 2019). KA is an anti-inflammatory in humans (Moon et al., 2001, Suthiram et al., 2023). The most distinguished attribute of KA is the compounds' ability to inhibit tyrosinase (polyphenol oxidase) activity (Choi et al., 2012, SCCP, 2021). KA is a chemo-sensitizing compound of complex III inhibitors that disrupts fungal mitochondrial respiration (Kim et al., 2013a). Additionally, KA elevated antioxidant responses (Khan et al., 2021) and displayed antitumor effects (Karakaya et al., 2019a). The effect of KA on mitochondrial functionality in humans is largely unexplored.

Humans require energy to sustain life; at the cellular level, this energy is produced in the mitochondria. Mitochondrial respiration involves a series of reactions that transfer chemical energy from consumed

food and oxygen into transmembrane electrochemical potential. The mitochondria facilitate the synthesis of ATP, calcium uptake, macromolecule biosynthesis and many other functions (Picard et al., 2018).

Complex I and III are known to solely produce mitochondrial reactive oxygen species (ROS) (Chouchani et al., 2014). Mitochondrial dysfunction due to excessive ROS production hinders the organism's ability to sustain life and can lead to disorders and diseases. Mitochondrial ROS benefits cells when used as a signalling molecule but high levels of ROS causes DNA, protein, and lipid damage. Oxidative stress is caused by high ROS levels and can be detrimental to cells (Schieber and Chandel, 2014, Mailloux, 2020).

Cells activate nuclear factor erythroid 2-related factor-2 (Nrf-2) to enhance the transcription of antioxidant response proteins. Nrf-2 interacts with Kelch-like-ECH-associated protein 1 (KEAP-1). Under conditions of high ROS, cysteine residues are oxidised resulting in the dissociation of KEAP-1 from Nrf-2. Nrf-2 is free to translocate to the nucleus and form heterodimer bonds with bZIP (basic region leucine zipper)-type transcription factors, such as small Maf proteins, and antioxidant-responsive elements (ARE) (Sporn and Liby, 2012, Schieber and Chandel, 2014).

When antioxidant responses are insufficient or bypassed, LON protease plays a key role in the removal of oxidised proteins in the mitochondria (Bota and Davies, 2002, Ngo and Davies, 2007). Sirtuins require nicotinamide adenine dinucleotide (NAD^+) to deacetylate mitochondrial proteins (Carrico et al., 2018). Sirtuin 3 initiates global acetylation of mitochondrial proteins (Hebert et al., 2013). Additionally, Sirtuin 3 maintains low levels of ROS allowing efficient metabolic and genetic functioning. Sirtuin 3 deacetylates LON protease resulting in the inhibition of tumorigenesis, therefore, regulating the lysosomal degradation of LON protease (Wu et al., 2023).

There is limited data on the effect of KA on the mitochondria, specifically assessing key mitochondrial proteins and oxidative markers. Therefore, this study aimed to determine the effect of KA on mitochondria in SK-MEL-1 melanoma cells. This was done by evaluating the mitochondrial output, oxidative damage to macromolecules, membrane integrity, antioxidant responses, and mitochondrial homeostasis proteins.

Materials and Methods

Cell culture and treatment

SK-MEL-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% foetal calf serum, 1% penicillin-streptomycin-fungizone, and 1% L-glutamine in 25 cm^3 cell culture flasks. The cells were grown in a 37°C humidified incubator with a constant supply of 5% CO_2.

The stock solution of KA (20 mg/ml, Sigma Aldrich (CAS No 501-30-4)) was prepared in 0.1 M phosphate-buffered saline (PBS).

Methyl thiazol tetrazolium (MTT) cytotoxicity assay

An MTT assay was performed on SK-MEL-1 cells to determine the effect of KA on mitochondrial output following 24 h exposure. In a 96-well microtiter plate, SK-MEL-1 (20,000 cells/well) were seeded and treated with KA at various doses (0 – 500 µg/ml). MTT salt solution [20 µl; 5 mg/ml in 0.1 M PBS] and RPMI 1640 (100 µl) were added to the cells and incubated (37ºC, 4 h). The MTT salt was removed from each well and dimethyl sulfoxide (100 µl/well) was added to solubilise the formazan crystals and incubated for 1 h at 37ºC. A Biotek µQuant Plate reader (Winooski, VT, USA) measured the optical density at 570 nm at a reference wavelength of 690 nm. GraphPad Prism V5.0 was used to analyse the absorbances obtained and concentrations [0, 25, 100, 500 µg/ml] were used for the following assays.

ATP luminometry

Intracellular ATP levels are measured using an ATP Cell Titre Glo® luminometry assay kit (Promega #G7570, Madison, USA). In a white 96-well microtiter plate, SK-MEL-1 cells (20,000 cells in 50 µl of 0.1 M PBS in triplicate) were seeded and incubated with 20 µl of ATP Cell Titre Glo® reagent (30 min, room temperature (RT) in the dark). Luminescence was quantified and expressed as relative light units (RLU) using a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). RLU is proportional to intracellular ATP levels present in the sample.

Lipid peroxidation assay

The biomarker of lipid peroxidation, malondialdehyde (MDA), was measured using the thiobarbituric acid reactive substances (TBARS) assay. The respective supernatants (200 µl) were added in test tubes along with 0.1 M TBA/BHT solution (400 µl), 2% H_3PO_4 (200 µl), and 7% H_3PO_4 (200 µl). Additionally, a positive control containing 1% MDA (1 µl) and a negative control containing 3 mM HCl (400 µl) were prepared. Each sample was vortexed and 200 µl of 1 M HCl was added to lower the pH of the samples to 1.5. Samples were heated (100°C, 15 min) and allowed to cool to RT before butanol (1,500 µl) was added to each sample. The samples were vortexed and incubated to separate into two phases. The butanol phase (100 µl) was added to a 96-well microtiter plate in triplicate. The absorbance was measured using a Biotek µQuant spectrophotometer (Winooski, VT, USA) at 532 nm with a reference wavelength of 600 nm. MDA concentration (µM) was calculated by dividing the mean optical density by the absorption coefficient (156 mM^{-1}).

Protein damage assay

Protein isolation was performed as previously described by Suthiram et al. (2023). To separate insoluble material, samples were incubated (37ºC, 10 min) and centrifuged (2,000 ×g, 10 min, RT). The sample supernatant (100 µl) was added in triplicate into a 96-well microtiter plate (100 µl) and the absorbance was measured at 370 nm using a spectrophotometer. The average absorbance obtained from the blank was subtracted from the sample absorbance to determine the corrected absorbance. The protein carbonyl concentration was obtained by dividing the corrected absorbance calculated by the 2,4-Dinitrophenol's (DNP's) extinction coefficient (22,000 $l.M^{-1}.cm^{-1}$). The results were expressed in nanomoles per milligram units.

Protein isolation, quantification, and expression (Western Blot)

Crude protein was isolated from treated SK-MEL-1 cells using CytoBuster™ reagent (200 µl, Novagen, 71009) supplemented with protease (Roche, 05892791001) and phosphatase inhibitors (Roche, 04906837001). Treated cells and the control were incubated on ice for 30 min. The cells were mechanically lysed and standardised to 1.5 mg/ml using a bicinchoninic acid assay. To each sample, 50 µl of Laemmli buffer consisting of dH_2O, 0.5 M Tris-Cl (6.8), bromophenol blue, β-mercaptoethanol, glycerol and 10% SDS was added and boiled for 5 min at 100ºC. Samples were electrophoresed in a 4% stacking gel and 7.5% resolving gel at 150 V (Bio-Rad compact power supply) for 1.5 h. Using a Trans-Blot Turbo System (Bio-Rad), the proteins were transferred onto nitrocellulose membranes (20 V, 30 min). To prevent non-specific binding, the membranes were incubated with 5% BSA for 1 h. Membranes were probed with primary antibodies (1:1,000, 1 h, RT) against LON protease/ PRSS15 (28020S, Cell Signalling Technology), Nrf-2 (ab76026, Abcam), and Sirtuin 3 (ab217319, Abcam) and then incubated overnight at 4ºC. The membranes were washed five times (10 min each, RT) with Tween 20 Tris-buffer saline [TTBS; 150 mM NaCl, 3 mM KCl, 25 mM Tris, 0.05% Tween 20, dH_2O, pH 7.5]. Horse-radish peroxidase (HRP)-conjugated secondary antibody goat anti-rabbit (7074, Cell Signalling Technology) was added to the membrane and incubated (1:5,000, 2 h, RT). Band density analysis was carried out using Invitrogen™ iBright™ imaging systems and analysis software. Results were expressed as relative band density (RBD).

Statistical analysis

All statistical analyses were carried out using GraphPad Prism version 5.0 (GraphPad Prism Software Inc.). One-way analysis of variance (ANOVA) with Tukey multiple comparison tests (95% CI) was used to analyse data sets. The results were expressed as the mean ± standard deviation (SD) (n = 3). Statistical significance was considered at $p < 0.05$.

Results

The effect of KA on mitochondrial output in melanoma cells

The effect of KA on the mitochondria output was assessed using the MTT assay and ATP luminometry over a 24 h period. We first assessed cell viability using the MTT assay which measures the cell's metabolic ability to generate reducing equivalents. The percentage of cell viability increased (p = 0.0001, Figure 3.1a). Furthermore, ATP levels were significantly depleted relative to the control. Notably, the highest decrease was at the 100 µg/ml concentration (p = 0.0002, Figure 3.1b).

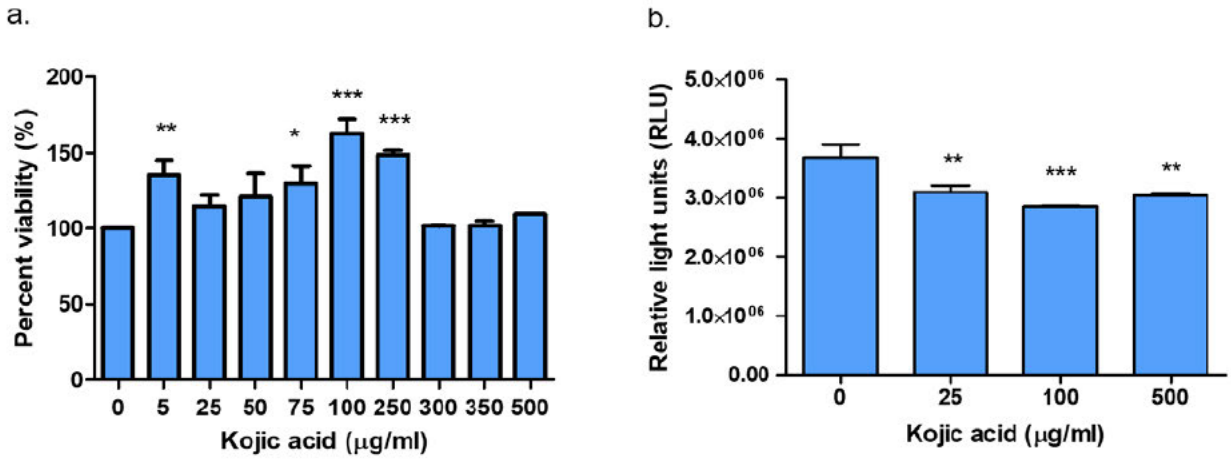

Figure 3.1: Mitochondrial output was assessed over a 24 h period; a) KA significantly increased cell viability and b) Intracellular ATP was significantly depleted in KA-treated cells. Results are represented as mean fold-change ± SD (n = 3), where ***p < 0.001, **p < 0.01 and *p < 0.05.

Modulation of KA-induced oxidative damage and antioxidant response

The effect of KA on oxidative damage was determined in SK-MEL-1 cells and the results depicted an increase in MDA production (p < 0.0001, Figure 3.2). The antioxidant response protein, Nrf-2, was significantly increased at all concentrations (p = 0.0002, Figure 3.3).

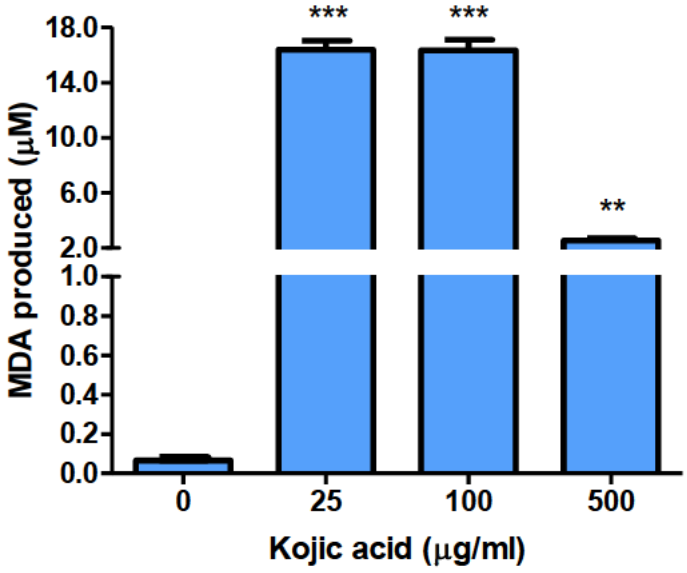

Figure 3.2: MDA levels were significantly increased in KA-treated cells following a 24 h treatment. Results are represented as mean fold-change ± SD ($n = 3$), where ***$p < 0.001$ and **$p < 0.01$.

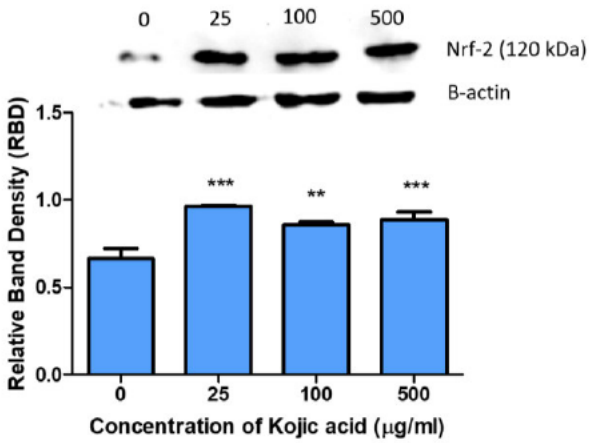

Figure 3.3: Nrf-2 expression was significantly increased in KA-treated cells following a 24 h treatment. Results are represented as mean fold-change ± SD ($n = 3$), where ***$p < 0.001$ and **$p < 0.01$.

Maintenance of mitochondrial homeostasis in melanoma cells

Mitochondrial homeostasis was assessed over a 24 h treatment period. Macromolecules are vulnerable to damage in oxidative conditions, so concentrations of protein carbonyls were analysed. The results showed an increase in protein carbonyls at all concentrations ($p < 0.0001$, Figure 3.4). The expression of LON protease, which plays a key role in removing oxidatively damaged protein and regulating mitochondrial pathways, was also examined. The results revealed an increase in LON protease expression at all concentrations ($p = 0.0023$, Figure 3.5a). Sirtuin 3, which helps maintain mitochondrial integrity by regulating LON protease, was also investigated. Sirtuin 3 expression was decreased at 25 µg/ml and no significant change at 100 and 500 µg/ml following treatment ($p = 0.0169$, Figure 3.5b).

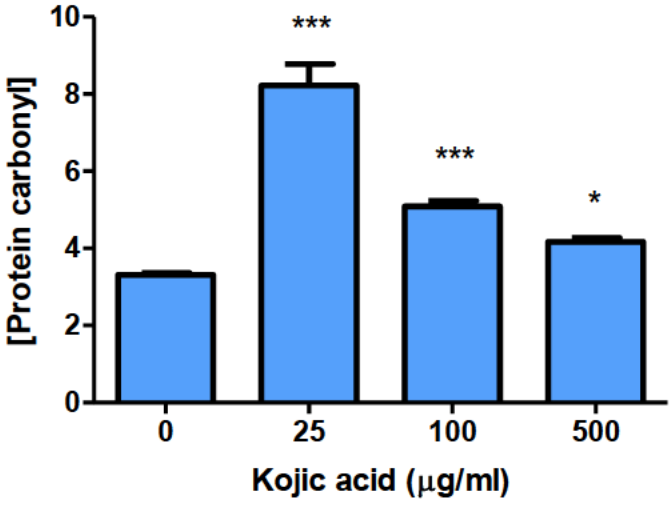

Figure 3.4: Protein carbonyl levels were significantly increased in KA-treated cells following a 24 h treatment. Results are represented as mean fold-change ± SD (n = 3), where ***p < 0.001 and *p < 0.05.

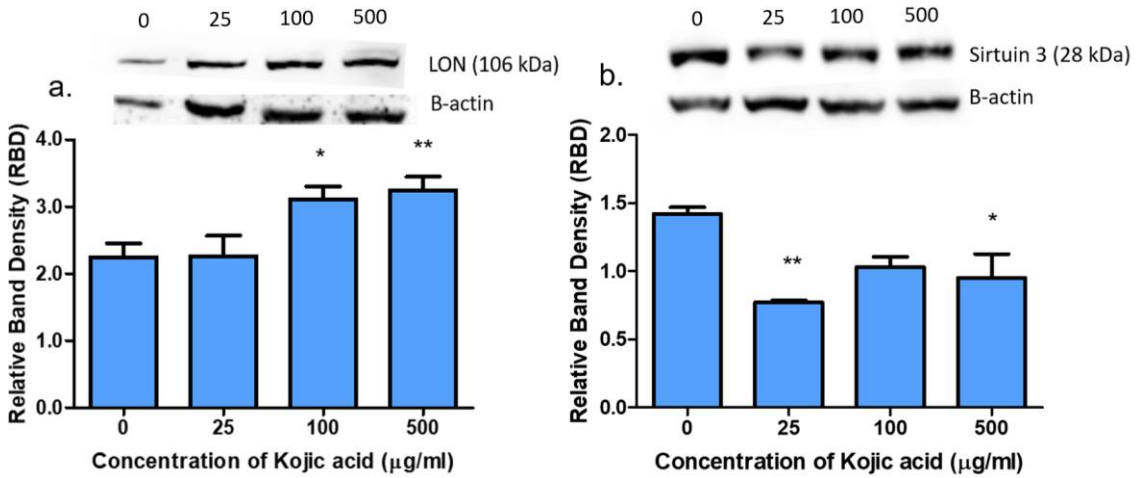

Figure 3.5: KA altered responses to protein damage in KA-treated cells following a 24 h treatment; a) LON protease expression was significantly increased and b) Sirtuin 3 expression was significantly decreased. Results are represented as mean fold-change ± SD (n = 3), where **p < 0.01 and *p < 0.05.

Discussion

The mitochondria are associated with melanocyte survival and melanocyte-keratinocyte communication in melanogenesis (Kaushik et al., 2023). Organelles such as the mitochondria are susceptible to oxidative damage which can lead to several skin conditions. KA is a well-known tyrosinase inhibitor which alters melanin production in skin cells (Cabanes et al., 2011, Karakaya et al., 2019b). This study investigated the impact of KA treatment on melanoma cells in terms of mitochondrial stress and the relevant antioxidant responses. This study bears importance due to KA's growing use in the cosmetics industry and the emergence of new and innovative applications in the medical industry. Studies to date, have assessed the chemo-sensitizing nature of complex III inhibitors in the mitochondrial electron transport chain in fungi (Kim et al., 2013a); however, the evaluation of mitochondrial stress in melanoma (SK-MEL-1) cells remains unexplored. Furthermore, it has been suggested that KA has anti-tumour potential (Higa et al., 2007, Zilles et al., 2022), albeit the exact mechanism is still unknown. The study has the potential to shed light on the mitochondrial effect of KA in melanoma cells over 24 h – the usual time period for the use of skincare products.

To evaluate the cytotoxic ability of KA, we used SK-MEL-1 cells, a tumorigenic cell line. The study found an increase in cell viability, an undesirable result due to the cell line's cancerous nature. The study utilised an MTT assay which assessed the cell's ability to metabolise MTT salt into insoluble

formazan product by the mitochondrial succinate dehydrogenase enzyme. Succinate dehydrogenase is found in complex II in the electron transport chain (Rustin et al., 2002). Concentrations obtained from Figure 3.1a were used to further assess mitochondrial output by measuring intracellular ATP levels. It was determined that there was a decrease in ATP levels after the 24 h incubation period (Figure 3.1b). Notably, the concentration that resulted in the highest cell survival also had the largest reduction in intracellular ATP. No studies have reported on the mitochondrial effect of KA in humans. Previous studies found interaction of KA at complex III inhibitors specifically in filamentous fungi (Kim et al., 2013a). Our study showed that KA could be inhibiting the ETC at complex III which in turn affects complex V. ATP synthesis occurs at complex V (Rustin et al., 2002, Chen et al., 2023). Complex V may not occur due to the disruption of the proton gradient leading to decreased ATP synthesis. This accounts for an increase in cell viability and decrease in ATP levels.

While cell survival remains unaffected, a decrease in ATP turnover can negatively affect processes such as macromolecule repair. To gauge the full extent of the effect of depleted ATP, we analysed the level of oxidative damage and the resulting initiation of antioxidant responses. Oxidative stress marker concentrations (MDA) were increased at lower concentrations (Figure 3.2). Previous studies state that KA is an antioxidant (Niwa and Akamatsu, 1991, Lajis et al., 2012a, Lobato et al., 2020). In the presence of oxidative stress, antioxidant responses are initiated to reduce damage to susceptible macromolecules. One such defence is initiated by Nrf-2 which activates proteins to alleviate oxidative stress. KA facilitated the increase of Nrf-2 protein expression at concentrations of elevated MDA production (Figure 3.3). This antioxidant response could be due to KA's chelation of cysteine residues resulting in Nrf-2 release. Additionally, SK-MEL-1 cells are a melanin-producing cell line. Elevated Nrf-2 expression results in reduced melanin production due to decreased tyrosinase and tyrosinase related-protein 1 (TRP-1) activity (Shin et al., 2014). KA is known to inhibit tyrosinase activity. The results imply that KA increases Nrf-2 activity resulting in a decrease in melanin production. More research needs to be carried out to fully describe KA's role in facilitating melanin reduction via the Nrf-2 antioxidant pathway.

Proteins are highly sensitive to oxidative stress causing aggregated, modified, or fragmented side chains. These proteins are involved in the onset and progression of disease (Estévez and Xiong, 2019). The use of KA caused protein oxidation at lower concentrations (Figure 3.4). Protein carbonyls produced correlated with the high MDA concentrations levels. KA forms salts by chelating metals (Beelik, 1956, Coupland and Niehaus, 1987, Saeedi et al., 2019). The compound's weak acid properties could explain the oxidation of proteins. KA forms salts with metals such as sodium, copper, zinc, calcium, nickel, and cadmium (Beelik, 1956, Coupland and Niehaus, 1987, Saeedi et al., 2019). Another study found that KA binds to iron (III) and aluminium (III) to form complexes (Nurchi et al., 2011b). These metalloproteins are found in the body and play a crucial role in several pathways.

LON protease is activated by oxidative stress (Ngo and Davies, 2009). LON protease is an ATP-dependent serine protease that functions as a chaperone that removes damaged proteins and regulates mitochondrial pathways (Pinti et al., 2016). LON protease expression was significantly increased at all concentrations due to increased protein carbonyls (Figure 3.5a). Decreased ATP levels could cause deficient LON protease functionality resulting in increased protein carbonyls. LON protease is a stress response protein that is regulated by Sirtuin 3 (Gibellini et al., 2014).

Sirtuin 3 is a significant deacetylase found in the mitochondria that play a crucial role in regulating various processes such as mitochondrial metabolism, suppressing inflammation and oxidative stress, regulating apoptosis and autophagy, and maintaining mitochondrial homeostasis (Schwer and Verdin, 2008b, Anderson et al., 2014, Torrens-Mas et al., 2019, Zhang et al., 2020). Sirtuin 3 can display pro- and anti-tumour effects. Sirtuin 3 can deacetylate tumour suppressor transcription factor (p53). When p53 is deacetylated, it can lead to its degradation, which in turn leads to the proliferation of cancer cells (Xiong et al., 2018). Conversely, mitochondrial activity is stimulated by Sirtuin 3 which initiates the deacetylation of pyruvate dehydrogenase. As a result, cancer cell proliferation is reduced (Yapryntseva et al., 2022). NAD^+ serves as a substrate for Sirtuin 3 deacetylation reactions (Carrico et al., 2018). NAD^+ catalyses the transfer of electrons in oxidation-reduction reactions involved in glycolysis and the citric acid (TCA) cycle. The NAD^+/NADH ratio dictates the efficiency of energy production (Stein and Imai, 2012). KA potentially disrupted mitochondrial functionality by reducing NAD^+ levels. This, in turn, decreased Sirtuin 3 expression and could account for depleted ATP levels following treatment. Sirtuin 3 is activated by oxidative stress and facilitates superoxide dismutase (SOD) antioxidant activity (Chen et al., 2011). Decreased Sirtuin 3 expression leads to an increase in ROS production.

Sirtuin 3 expression was decreased at all concentrations (Figure 3.5b). This is expected due to the inversely proportional relationship between Sirtuin 3 and LON protease. Sirtuin 3 has a zinc-binding motif (Zhang et al., 2020). Sirtuin 3 structure may play a vital role in the suppression of the protein's expression. Moreover, Nrf-2 can act as a transcriptional regulator of LON expression (Pinti et al., 2011). Increased Nrf-2 expression could have limited Lon protease functionality, resulting in an increase of protein oxidation.

Conclusion

KA causes damage to proteins which leads to stress in the mitochondria. However, KA was not cytotoxic to SK-MEL-1 melanoma cells. The study showed KA's ability to interact with key mitochondrial proteins. The efficient functioning of mitochondria in skin cells is highly regulated due to its role as a barrier to entry and the production of melanin. Perturbed mitochondrial function and increased protein oxidation can lead to aberrant processes within the cell. This study supplies sufficient

data to suggest concentration-dependent changes in mitochondrial protein expression. This highlights the need for regulation of the concentration of KA in cosmetic products. One limitation of our study was that we only conducted an *in vitro* assessment of KA's impact on mitochondrial stress. Although the study findings are relevant to the use of KA, validation of results should be carried out in an *in vivo* model which simulates a more accurate environment. Further research should be conducted on how KA contributes to the Nrf-2 antioxidant pathways and its effect on reducing melanin production. The mitochondria have been implicated in many diseases in humans. This discovery brings focus to the levels of KA present in skin care products and how it impacts the functioning of mitochondria. The study highlights the potential of KA in diseases related to mitochondrial dysfunction and homeostasis.

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Authors contributions

Conceptualisation: KTS, TG, and AC. Methodology: KTS. Formal analysis and investigation: KTS. Writing- original draft: KTS. Review and editing: KTS, TG, and AC. Supervision: TG and AC.

Disclosure Statement

The authors declare no conflict of interest.

References

ANDERSON, K. A., GREEN, M. F., HUYNH, F. K., WAGNER, G. R. & HIRSCHEY, M. D. 2014. SnapShot: Mammalian Sirtuins. *Cell,* 159**,** 956-956.e1. DOI: 10.1016/j.cell.2014.10.045.

BEELIK, A. 1956. Kojic acid. *Advances in Carbohydrate Chemistry and Biochemistry,* 48**,** 145-183.

BOTA, D. A. & DAVIES, K. J. 2002. Lon protease preferentially degrades oxidized mitochondrial aconitase by an ATP-stimulated mechanism. *Nature Cell Biology,* 4**,** 674-80. DOI: 10.1038/ncb836.

BURDOCK, G. A., SONI, M. G. & CARABIN, I. G. 2001. Evaluation of health aspects of kojic acid in food. *Regulatory Toxicology and Pharmacology,* 33**,** 80-101. DOI: 10.1006/rtph.2000.1442.

BURNETT, C. L., BERGFELD, W. F., BELSITO, D. V., HILL, R. A., KLAASSEN, C. D., LIEBLER, D. C., MARKS, J. G., SHANK, R. C., SLAGA, T. J., SNYDER, P. W. & ANDERSEN, F. A. 2010. Final Report of the Safety Assessment of Kojic Acid as Used in Cosmetics. *International Journal of Toxicology,* 29**,** 244-273. DOI: 10.1177/1091581810385956.

CABANES, J., CHAZARRA, S. & GARCIA-CARMONA, F. 2011. Kojic Acid, a Cosmetic Skin Whitening Agent, is a Slow-binding Inhibitor of Catecholase Activity of Tyrosinase. *Journal of Pharmacy and Pharmacology,* 46**,** 982-985. DOI: 10.1111/j.2042-7158.1994.tb03253.x.

CARRICO, C., MEYER, J. G., HE, W., GIBSON, B. W. & VERDIN, E. 2018. The mitochondrial acylome emerges: proteomics, regulation by sirtuins, and metabolic and disease implications. *Cell metabolism,* 27**,** 497-512.

CHEN, W., ZHAO, H. & LI, Y. 2023. Mitochondrial dynamics in health and disease: mechanisms and potential targets. *Signal Transduction and Targeted Therapy,* 8**,** 333. DOI: 10.1038/s41392-023-01547-9.

CHEN, Y., ZHANG, J., LIN, Y., LEI, Q., GUAN, K.-L., ZHAO, S. & XIONG, Y. 2011. Tumour suppressor SIRT3 deacetylates and activates manganese superoxide dismutase to scavenge ROS. *EMBO reports,* 12**,** 534-541. DOI: https://doi.org/10.1038/embor.2011.65.

CHOI, H., KIM, K., HAN, J., CHOI, H., JIN, S. H., LEE, E. K., SHIN, D. W., LEE, T. R., LEE, A.-Y. & NOH, M. 2012. Kojic acid-induced IL-6 production in human keratinocytes plays a role in its anti-melanogenic activity in skin. *Journal of Dermatological Science,* 66**,** 207-215. DOI: https://doi.org/10.1016/j.jdermsci.2012.03.002.

CHOUCHANI, E. T., PELL, V. R., GAUDE, E., AKSENTIJEVIĆ, D., SUNDIER, S. Y., ROBB, E. L., LOGAN, A., NADTOCHIY, S. M., ORD, E. N. & SMITH, A. C. 2014. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature,* 515**,** 431-435.

COUPLAND, K. & NIEHAUS, W. G. 1987. Effect of nitrogen supply, Zn2+, and salt concentration on kojic acid and versicolorin biosynthesis by *Aspergillus parasiticus*. *Experimental Mycology,* 11**,** 206-213. DOI: https://doi.org/10.1016/0147-5975(87)90006-5.

ERMIS, N., ZARE, N., DARABI, R., ALIZADEH, M., KARIMI, F., SINGH, J., SHAHIDI, S.-A., DRAGOI, E. N., CAMARADA, M. B. & BAGHAYERI, M. 2023. Recent advantage in electrochemical monitoring of gallic acid and kojic acid: a new perspective in food science. *Journal of Food Measurement and Characterization***,** 1–10. DOI: 10.1007/s11694-023-01881-0.

ESTÉVEZ, M. & XIONG, Y. 2019. Intake of Oxidized Proteins and Amino Acids and Causative Oxidative Stress and Disease: Recent Scientific Evidences and Hypotheses. *Journal of Food Science,* 84**,** 387-396. DOI: https://doi.org/10.1111/1750-3841.14460.

GIBELLINI, L., PINTI, M., BERETTI, F., PIERRI, C. L., ONOFRIO, A., RICCIO, M., CARNEVALE, G., BIASI, S., MILENA, N., TORELLI, F., BORALDI, F., POL, A. & COSSARIZZA, A. 2014. Sirtuin 3 interacts with Lon protease and regulates its acetylation state. *Mitochondrion,* 18**,** 76-81. DOI: 10.1016/j.mito.2014.08.001.

HEBERT, A. S., DITTENHAFER-REED, K. E., YU, W., BAILEY, D. J., SELEN, E. S., BOERSMA, M. D., CARSON, J. J., TONELLI, M., BALLOON, A. J. & HIGBEE, A. J. 2013. Calorie restriction and SIRT3 trigger global reprogramming of the mitochondrial protein acetylome. *Molecular cell,* 49**,** 186-199.

HIGA, Y., KAWABE, M., NABAE, K., TODA, Y., KITAMOTO, S., HARA, T., TANAKA, N., KARIYA, K. & TAKAHASHI, M. 2007. Kojic acid -absence of tumor-initiating activity in rat liver, and of carcinogenic and photo-genotoxic potential in mouse skin. *The Journal of Toxicological Sciences,* 32**,** 143-159. DOI: 10.2131/jts.32.143.

KARAKAYA, G., ERCAN, A., ÖNCÜL, S. & AYTEMİR, M. D. 2019a. Kojic acid derivatives as potential anticancer agents: Synthesis and cytotoxic evaluation on A375 human malignant melanoma cells. *Journal of Research in Pharmacy,* 23**,** 596-607. DOI: 10.12991/jrp.2019.167.

KARAKAYA, G., TÜRE, A., ERCAN, A., ÖNCÜL, S. & AYTEMIR, M. D. 2019b. Synthesis, computational molecular docking analysis and effectiveness on tyrosinase inhibition of kojic acid derivatives. *Bioorganic Chemistry,* 88**,** 102950. DOI: https://doi.org/10.1016/j.bioorg.2019.102950.

KAUSHIK, H., KUMAR, V. & PARSAD, D. 2023. Mitochondria–Melanocyte cellular interactions: An emerging mechanism of vitiligo pathogenesis. *Journal of the European Academy of Dermatology and Venereology***,** 1-12. DOI: https://doi.org/10.1111/jdv.19019.

KHAN, A., PARK, T. J., IKRAM, M., AHMAD, S., AHMAD, R., JO, M. G. & KIM, M. O. 2021. Antioxidative and Anti-inflammatory Effects of Kojic Acid in Aβ-Induced Mouse Model of Alzheimer's Disease. *Molecular Neurobiology,* 58**,** 5127-5140. DOI: 10.1007/s12035-021-02460-4.

KIM, J. H., CAMPBELL, B. C., CHAN, K. L., MAHONEY, N. & HAFF, R. P. 2013. Synergism of Antifungal Activity between Mitochondrial Respiration Inhibitors and Kojic Acid. *Molecules,* 18**,** 1564-1581.

KIM, J. H., CHANG, P.-K., CHAN, K. L., FARIA, N. C. G., MAHONEY, N., KIM, Y. K., MARTINS, M. D. L. & CAMPBELL, B. C. 2012. Enhancement of commercial antifungal agents by Kojic Acid. *International Journal of Molecular Sciences,* 13**,** 13867-13880. DOI: 10.3390/ijms131113867.

LAJIS, A. F. B., HAMID, M. & ARIFF, A. B. 2012. Depigmenting effect of Kojic acid esters in hyperpigmented B16F1 melanoma cells. *Journal of Biomedicine & Biotechnology,* 2012**,** 1-9. DOI: 10.1155/2012/952452.

LEE, H. F., BOLTJES, B. & EISENMAN, W. 1950. Kojic Acid as an Inhibitor of Tubercle Bacilli. *American Review of Tuberculosis and Pulmonary Diseases,* 61**,** 738-741.

LOBATO, C. C., ORDOÑEZ, M. E., QUEIROZ, R. L., SANTOS, C. B. R. & BORGES, R. S. 2020. A comparative study between kojic acid and its methylated derivatives as antioxidant related to maltol and alomaltol. *Chemical Data Collections,* 28**,** 100464. DOI: https://doi.org/10.1016/j.cdc.2020.100464.

MAILLOUX, R. J. 2020. An Update on Mitochondrial Reactive Oxygen Species Production. *Antioxidants,* 9**,** 472.

MOON, K. Y., AHN, K. S., LEE, J. & KIM, Y. S. 2001. Kojic acid, a potential inhibitor of NF-kappaB activation in transfectant human HaCaT and SCC-13 cells. *Archives of Pharmacal Research,* 24**,** 307-311. DOI: 10.1007/bf02975097.

NAKAGAWA, M., KAWAI, K. & KAWAI, K. 1995. Contact allergy to kojic acid in skin care products. *Contact Dermatitis,* 32**,** 9-13. DOI: 10.1111/j.1600-0536.1995.tb00832.x.

NGO, J. K. & DAVIES, K. J. 2009. Mitochondrial Lon protease is a human stress protein. *Free Radical Biology and Medicine,* 46**,** 1042-8. DOI: 10.1016/j.freeradbiomed.2008.12.024.

NGO, J. K. & DAVIES, K. J. A. 2007. Importance of the Lon Protease in Mitochondrial Maintenance and the Significance of Declining Lon in Aging. *Annals of the New York Academy of Sciences,* 1119**,** 78-87. DOI: https://doi.org/10.1196/annals.1404.015.

NIWA, Y. & AKAMATSU, H. 1991. Kojic acid scavenges free radicals while potentiating leukocyte functions including free radical generation. *Inflammation,* 15**,** 303-315. DOI: 10.1007/BF00917315.

NURCHI, V. M., LACHOWICZ, J. I., CRISPONI, G., MURGIA, S., ARCA, M., PINTUS, A., GANS, P., NICLOS-GUTIERREZ, J., DOMÍNGUEZ-MARTÍN, A., CASTINEIRAS, A., REMELLI, M., SZEWCZUK, Z. & LIS, T. 2011. Kojic acid derivatives as powerful chelators for iron(III) and aluminium(III). *Dalton Trans,* 40**,** 5984-98. DOI: 10.1039/c1dt00005e.

PICARD, M., MCEWEN, B. S., EPEL, E. S. & SANDI, C. 2018. An energetic view of stress: Focus on mitochondria. *Frontiers in Neuroendocrinology,* 49**,** 72-85. DOI: https://doi.org/10.1016/j.yfrne.2018.01.001.

PINTI, M., GIBELLINI, L., DE BIASI, S., NASI, M., ROAT, E., O'CONNOR, J. E. & COSSARIZZA, A. 2011. Functional characterization of the promoter of the human Lon protease gene. *Mitochondrion,* 11**,** 200-6. DOI: 10.1016/j.mito.2010.09.010.

PINTI, M., GIBELLINI, L., NASI, M., DE BIASI, S., BORTOLOTTI, C. A., IANNONE, A. & COSSARIZZA, A. 2016. Emerging role of Lon protease as a master regulator of mitochondrial functions. *Biochimica et Biophysica Acta (BBA) - Bioenergetics,* 1857**,** 1300-1306. DOI: https://doi.org/10.1016/j.bbabio.2016.03.025.

RUSTIN, P., MUNNICH, A. & RÖTIG, A. 2002. Succinate dehydrogenase and human diseases: new insights into a well-known enzyme. *European Journal of Human Genetics,* 10**,** 289-291. DOI: 10.1038/sj.ejhg.5200793.

SAEEDI, M., ESLAMIFAR, M. & KHEZRI, K. 2019. Kojic acid applications in cosmetic and pharmaceutical preparations. *Biomedicine and Pharmacotherapy,* 110**,** 582-593. DOI: 10.1016/j.biopha.2018.12.006.

SCCP 2021. Opinion on Kojic acid. *European Commission***,** 1-85.

SCHIEBER, M. & CHANDEL, NAVDEEP S. 2014. ROS Function in Redox Signaling and Oxidative Stress. *Current Biology,* 24**,** R453-R462. DOI: https://doi.org/10.1016/j.cub.2014.03.034.

SCHWER, B. & VERDIN, E. 2008. Conserved metabolic regulatory functions of sirtuins. *Cell Metabolism,* 7**,** 104-12. DOI: 10.1016/j.cmet.2007.11.006.

SHIN, J.-M., KIM, M. Y., SOHN, K.-C., JUNG, S.-Y., LEE, H.-E., LIM, J. W., KIM, S., LEE, Y.-H., IM, M., SEO, Y.-J., KIM, C. D., LEE, J.-H., LEE, Y. & YOON, T.-J. 2014. Nrf2 Negatively Regulates Melanogenesis by Modulating PI3K/Akt Signaling. *PLOS ONE,* 9**,** e96035. DOI: 10.1371/journal.pone.0096035.

SPORN, M. B. & LIBY, K. T. 2012. NRF2 and cancer: the good, the bad and the importance of context. *Nature Reviews Cancer,* 12**,** 564-571.

STEIN, L. R. & IMAI, S.-I. 2012. The dynamic regulation of NAD metabolism in mitochondria. *Trends in Endocrinology & Metabolism,* 23**,** 420-428. DOI: 10.1016/j.tem.2012.06.005.

SUTHIRAM, K. T., GHAZI, T., ABDUL, N. S. & CHUTURGOON, A. A. 2023. Kojic acid induces oxidative stress and anti-inflammatory effects in human hepatocellular carcinoma (HepG2) cells. *Toxicon,* 232**,** 107221. DOI: https://doi.org/10.1016/j.toxicon.2023.107221.

TORRENS-MAS, M., HERNÁNDEZ-LÓPEZ, R., PONS, D.-G., ROCA, P., OLIVER, J. & SASTRE-SERRA, J. 2019. Sirtuin 3 silencing impairs mitochondrial biogenesis and metabolism in colon cancer cells. *American Journal of Physiology-Cell Physiology,* 317**,** C398-C404. DOI: 10.1152/ajpcell.00112.2019.

WU, L., YAN, X., SUN, R., MA, Y., YAO, W., GAO, B., ZHANG, Q., YOU, J., WANG, H., HAN, Q. & SUN, X. 2023. Sirt3 restricts tumor initiation via promoting LONP1 deacetylation and K63 ubiquitination. *Journal of Translational Medicine,* 21**,** 81. DOI: 10.1186/s12967-023-03925-x.

WU, Y., SHI, Y. G., ZENG, L. Y., PAN, Y., HUANG, X. Y., BIAN, L. Q., ZHU, Y. J., ZHANG, R. R. & ZHANG, J. 2019. Evaluation of antibacterial and anti-biofilm properties of kojic acid against five food-related bacteria and related subcellular mechanisms of bacterial inactivation. *Food Science and Technology International,* 25**,** 3-15. DOI: 10.1177/1082013218793075.

XIONG, Y., WANG, L., WANG, S., WANG, M., ZHAO, J., ZHANG, Z., LI, X., JIA, L. & HAN, Y. 2018. SIRT3 deacetylates and promotes degradation of P53 in PTEN-defective non-small cell lung cancer. *Journal of Cancer Research and Clinical Oncology,* 144**,** 189-198. DOI: 10.1007/s00432-017-2537-9.

YAPRYNTSEVA, M. A., MAXIMCHIK, P. V., ZHIVOTOVSKY, B. & GOGVADZE, V. 2022. Mitochondrial sirtuin 3 and various cell death modalities. *Frontiers in Cell and Developmental Biology,* 10**,** 947357. DOI: 10.3389/fcell.2022.947357.

ZHANG, J., XIANG, H., LIU, J., CHEN, Y., HE, R. R. & LIU, B. 2020. Mitochondrial Sirtuin 3: New emerging biological function and therapeutic target. *Theranostics,* 10**,** 8315-8342. DOI: 10.7150/thno.45922.

ZILLES, J. C., DOS SANTOS, F. L., KULKAMP-GUERREIRO, I. C. & CONTRI, R. V. 2022. Biological activities and safety data of kojic acid and its derivatives: A review. *Experimental Dermatology*, 31**,** 1500-1521. DOI: https://doi.org/10.1111/exd.14662.

CHAPTER 4

Kojic acid induces inflammatory responses independent of the Akt and MAPK signalling pathways

Kojic acid is known to interact with inflammatory mediators. Chapter 4 evaluated the effect of KA macromolecule damage and mitochondrial stress on protein kinase pathways and inflammation. Due to KA's use in cosmetics, we evaluated the impact of KA treatment on NFκB and NLRP3 inflammasome activation in SK-MEL-1 cells.

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Kojic acid induces inflammatory responses independent of the Akt and MAPK signalling pathways

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Abstract

Inflammation and protein kinases are known mediators of inflammatory conditions in the epidermis. Kojic acid is widely used to treat hyperpigmentation due to its chelating properties. Due to the growing popularity of its use, we assessed the effect of kojic acid on inflammation through common cell signalling pathways namely Protein kinase B/Akt and Mitogen activated protein kinase (MAPK). Protein and gene expression of Akt, phosphorylated glycogen synthesis kinase 3β (GSK3β), MAPK (p38 and ERK1/2), NFκB, IκB, NLRP3, interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) were assessed using Western Blot and qPCR. Intracellular caspase 1 levels were measured using luminometry. KA decreased the protein kinase expression of ERK1/2 ($p < 0.0001$), p38 ($p < 0.0001$), total *Akt* ($p = 0.0149$), phosphorylated Akt ($p < 0.0001$) and phosphorylated *GSK3β* ($p = 0.0004$). Inflammatory mediators such as NFκB and the NLRP3 inflammasome were upregulated in a concentration-dependent manner. NFκB gene and protein expression were increased ($p = 0.0007$ and $p = 0.3648$, respectively) by KA. The inhibitor of NFκB (*IκB*) was increased at high concentrations ($p = 0.1758$). Inflammasome machinery caspase 1 ($p = 0.0168$) and *NLRP3* ($p = 0.2993$) expression was also increased which facilitated the increase of *IL-1β* ($p = 0.0205$) and *IL-6* ($p = 0.472$) release. Inflammatory markers and interleukins were increased although KA perturbed protein kinase signalling. The findings illustrate that KA is a potent inhibitor of protein kinases and induces inflammation in SK-MEL-1 cells.

Keywords: Kojic acid, inflammation, MAPK, protein kinase, NFκB, NLRP3 inflammasome

Introduction

Kojic acid (KA) is a chelator used extensively in the cosmetic industry due to its role in suppressing polyphenol oxidase (tyrosinase) activity (Lima et al., 2014, Azami et al., 2017). While KA has a wide range of applications, its role in tyrosinase inhibition, oxidative stress, inflammation, and cancer has been the most studied (Niwa and Akamatsu, 1991, Lee et al., 2019a, Wei et al., 2019, Lachowicz et al., 2022, Zilles et al., 2022).

Inflammation triggers and exacerbates many skin diseases. Regulating inflammatory mediators is crucial in preventing and treating these conditions as well as maintaining healthy skin. To better understand the role of KA in inflammation, we conducted an evaluation of its effects on several different factors, including protein kinase B (Akt), mitogen-activated protein kinase (MAPK), nuclear factor kappa beta (NFκB), and NLR family pyrin domain containing 3 (NLRP3) inflammasome.

The phosphatidyl-inositol-3 kinase (PI_3K) pathway involves serine/threonine kinases that maintain homeostasis in epidermal cells. It is associated with immune-related inflammatory diseases such as acne, atopic dermatitis, alopecia, psoriasis, and vitiligo (Park et al., 1997, Roy et al., 2023). The PI_3K structure contains a catalytic domain and a regulatory domain.

Activation of the PI_3K signalling pathway by PIP_3 results in recruitment of Akt to the plasma membrane. Subsequently, receptor-binding activates PI_3K, which in turn activates PDK-1 and Akt (Chamcheu et al., 2019). The Akt protein undergoes phosphorylation, which enables it to regulate genes that prevent cell death. Additionally, Akt has an impact on the expression of NFκB, a transcription factor involved in immune response (Roy et al., 2023). The activity of tyrosinase in cells is regulated through the PI_3K signalling pathway, which is mediated by the expression of the microphthalmia-associated transcription factor (MITF). MITF plays a crucial role in melanocyte differentiation, proliferation, survival, and pigmentation. Skin pigmentation is determined by the presence of tyrosinase, as well as the proteins tyrosinase-related protein-1 (TRP-1) and tyrosinase-related protein-2 (TRP-2). MITF dictates the expression of key melanin-regulating proteins. The Akt pathway enhances MITF expression, increasing melanogenesis (Yin et al., 2017). Moreover, Akt stabilizes β-catenin by phosphorylating and inactivating glycogen synthesis kinase-3β (GSK-3β). β-catenin, in turn, binds to the promoter region of the MITF gene, thereby increasing MITF expression. This increase in MITF expression leads to elevated expression of tyrosinase and other related proteins, which results in enhanced melanin synthesis (Zang et al., 2019, Roy et al., 2023).

The MAPKs are a group of kinases that catalyse gene expression, cell proliferation, and apoptosis (Espinosa-Diez et al., 2015). ERK1/2 and p38 are dual phosphorylated at tyrosine/threonine residues. The dual activation at threonine and tyrosine residues alters the folding of p38. The isoforms p38α and

p38β are highly expressed in epidermal cells (Liu et al., 2014a). MAP2Ks (MKK3 and MKK6) are responsible for the phosphorylation of p38 (Doza et al., 1995, Dérijard et al., 1995). Activation of ERK1/2 occurs through a three-step process involving MAPKKK, MAPKK, and MAPK (Coleman et al., 2004, Sivaprasad and Basu, 2008, Overmeyer and Maltese, 2011, Kim et al., 2014, Asati et al., 2016, Zaleśna et al., 2016, Kong et al., 2019, Lu and Malemud, 2019). Previous studies have found that phosphorylation of Akt, ERK, and p38 upregulates MITF expression, which increases melanin production.

The NFκB, MAPK, and Akt pathways activate cytokines and interleukins to stimulate immune and inflammatory responses (Jeon et al., 2013). NFκB resides in the cytoplasm, bound to an inhibitor of NFκB (IκB). Under normal conditions, NFκB remains inactive due to the binding of IκB. However, when the cell receives certain stimuli, such as infection or oxidative stress, IκB is degraded and NFκB is released. This allows NFκB to move into the nucleus and activate the transcription of genes involved in immune and inflammatory responses (Hayden and Ghosh, 2012, Jeon et al., 2013). NFκB is also controlled by the MAPK and PI_3K/Akt signalling pathways (Qi et al., 2012). MAPK and Akt signalling regulate cellular responses, and persistent activation can lead to inflammation and disease.

NLRP3 inflammasome regulates inflammatory responses and mediates interleukin-1 beta (IL-1β) maturation (Yoon et al., 2013). The NLRP3 inflammasome is composed of the NLRP3 protein, ASC adaptor, and pro-caspase 1 (Elliott and Sutterwala, 2015). It has been found that NFκB and MAPK signalling are crucial during the activation of the inflammasome (Fang et al., 2020). NFκB mediates the expression of tumour necrosis factor alpha (TNFα), IL-1β, interleukin-6 (IL-6) and interleukin-8 (IL-8) responses (Tak and Firestein, 2001, Fang et al., 2020). KA is known for its anti-inflammatory properties, but its mechanism remains unclear. In addition, KA studies on protein kinases are limited. We evaluated the gene and protein expression of key protein kinases and inflammatory mediators to better understand their role.

Materials and methods

Materials

The SK-MEL-1 cell line was obtained from the American Type Culture Collection (ATCC; Johannesburg, South Africa). Cell culture supplies were purchased from Lonza Biotechnology (Basel, Switzerland). Caspase 1 luminometry kits were sourced from Promega (Madison, WI, USA) and Western Blot reagents were purchased from Bio-Rad (California, United States). Unless otherwise stated, all additional reagents and consumables were purchased from Merck (Darmstadt, Germany).

Cell culture and treatment

SK-MEL-1 melanoma cells (1.0 x 10^6) were cultured in complete culture medium (CCM consisting of Roswell Park Memorial Institute (RPMI) 1640 medium, 10% foetal calf serum (FCS), 1% penicillin-streptomycin-fungizone, and 1% L-glutamine). A stock solution of KA (20 mg/ml) was prepared in 0.1 M phosphate-buffered saline (PBS) and the cells were incubated in a 37°C humidified incubator with a constant flow of 5% CO_2 at varying concentrations [0, 25, 100 and 500 µg/ml]. The concentrations were obtained from our previous work (Addendum D, Figure S1)

RNA isolation and quantitative PCR

Treated cells were washed in 0.1 M PBS and incubated in 500 µl Trizol and 500 µl 0.1 M PBS for 5 min at RT. The cells were mechanically lysed using a cell scraper and cell lysates were stored overnight (-80°C). Following incubation, 100 µl of chloroform was added to thawed cell lysates and centrifuged (12,000 x g, 4°C, 10 min). The RNA-containing aqueous phase was transferred into a new microcentrifuge tube and 100% cold isopropanol (250 µl) was added to each sample and incubated overnight (-80°C). Samples were centrifuged (12,000 x g, 4°C, 20 min) and the RNA pellets were washed with 75% cold ethanol (500 µl). Lastly, the samples were centrifuged (7,400 x g, 4°C, 15 min), the ethanol was removed, and the resultant pellets were air-dried (30 min, RT).

The air-dried pellets were resuspended in nuclease-free water (15 µl). Isolated RNA from each treatment was quantified using the Nanodrop 2000 spectrophotometer (Thermo-Fischer Scientific, Waltham, USA) and standardised to 1,000 ng/µl. To determine RNA purity, the A_{260}/A_{280} ratio was used. Complementary DNA (cDNA) was synthesized from standardised RNA using the Maxima™ H Minus cDNA synthesis kit (ThermoFisher Scientific, K1652). Thermocycler conditions were 25ºC for 10 min, 50ºC for 15 min, and 85ºC for 5 min.

The gene expression of *Akt*, *NFκβ, IκB*, *NLRP3*, *IL-1β,* and *IL-6* was investigated using the PowerUP SYBR® Green Master Mix (ThermoFisher Scientific, A25742) and primers. Reaction volumes were prepared using SYBR green (5 µl), nuclease-free water (2 µl), forward primer (1 µl), reverse primer (1 µl), and cDNA template (1 µl). Each reaction was carried out in triplicate.

The CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) was used to amplify samples using the following cycling conditions: initial denaturation (8 min, 95ºC) followed by 40 cycles of denaturation (15s, 95ºC), annealing (40s, temperatures in Table 4.1) and extension (30s, 72ºC). The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used to normalise gene expression. The ($2^{-\Delta\Delta Ct}$) method described by (Livak and Schmittgen, 2001) was used to assess the relative change in gene expression.

Table 4.1: The annealing temperatures and primer sequences of the respective genes of interest

Gene name	Primer	Sequence (5'-3')	Annealing temperature (°C)
Akt	Forward	GTGCCGCAAAAGGTCTTCATG	63
	Reverse	TGGACTACCTGCACTCGGAGAA	
NLRP3	Forward	CAGGTGTTGGAATTAGACAAC	56
	Reverse	TTCAGACAACCCCAGGTTCT	
IL-1β	Forward	CCACAGACCTTCAGGAGAATG	60
	Reverse	GTGCAGTTCAGTGATCGTACAGG	
IL-6	Forward	GGAAGGTTCAGGTTGTTTTCTGC	56
	Reverse	AAATTCGGTACATCCTCGACGG	
NFκβ	Forward	TGAACCGAAACTCTGGCAGCTG	63
	Reverse	CATCAGCTTGCAAAAGGAGCC	
IκB	Forward	CACTCCATCCTGAAGGCTACCAAC	59
	Reverse	CACACTTCAACAGGAGTGACACCAG	
GAPDH	Forward	ACCACAGTCCATGCCATCAC	Same as gene of interest
	Reverse	TCCACCACCCTGTTGCTGTA	

Intracellular caspase 1 luminometry assay

Luminometry was used to determine the effect of KA on caspase-1 activity. SK-MEL-1 cells (20,000 cells/well) were seeded in an opaque 96-well microtiter plate with 25 µl of the Caspase Glo® -1 reagents. The plate was incubated in the dark for 30 min at RT. Luminescence was measured using a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). The results were presented as relative light units (RLU).

Western Blot

Protein was obtained from SK-MEL-1 cells using CytoBuster™ reagent (200 µl, Novagen, 71009) supplemented with protease (Roche, 05892791001) and phosphatase inhibitors (Roche, 04906837001). Controls and treated cells were incubated on ice and mechanically lysed. The crude protein was standardised to 1.5 mg/ml using the bicinchoninic acid assay. To each sample, 50 µl of Laemmli buffer [dH_2O, 0.5 M Tris-Cl (6.8), bromophenol blue, β-mercaptoethanol, glycerol and 10% SDS] was added and boiled for 5 min at 100°C. Samples were electrophoresed in a 4% stacking gel and 7.5% resolving gel at 150V for 1.5 h. The proteins were transferred onto nitrocellulose membranes using a Trans-Blot Turbo System (Bio-Rad) (20V, 30 min). Bovine serum albumin (BSA) was used to prevent non-specific binding. The membranes were incubated with 5% BSA for 1 h. Membranes were incubated with

primary antibodies (anti-phospho-Akt (ab81283, Abcam), anti-phospho-GSK3β (9336, Cell Signalling), anti-p38 (#9212, Cell Signalling), anti-ERK1/2 (#9102, Cell Signalling), anti-NFκβ (#8242, Cell Signalling); 1:1,000) for 1 h at RT) and overnight at 4ºC. The membranes were washed five times with Tween 20 Tris-buffer saline (TTBS; 25 mM Tris, 0.05% Tween 20, 50 mM NaCl, 3 mM KCl, dH_2O, pH 7.5; 10 min each, RT). Horse-radish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit (7074, Cell Signalling); 1:5,000) was added to the membrane and incubated overnight at 4ºC. Membranes were quenched with 5% hydrogen peroxide (37°C, 30 min), washed with TTBS, blocked with 5% BSA and incubated in HRP-conjugated antibody for β-actin (A3854, Sigma-Aldrich). Band density analysis was performed using Invitrogen™ iBright™ imaging systems and analysis software. Results were expressed as relative band density (RBD).

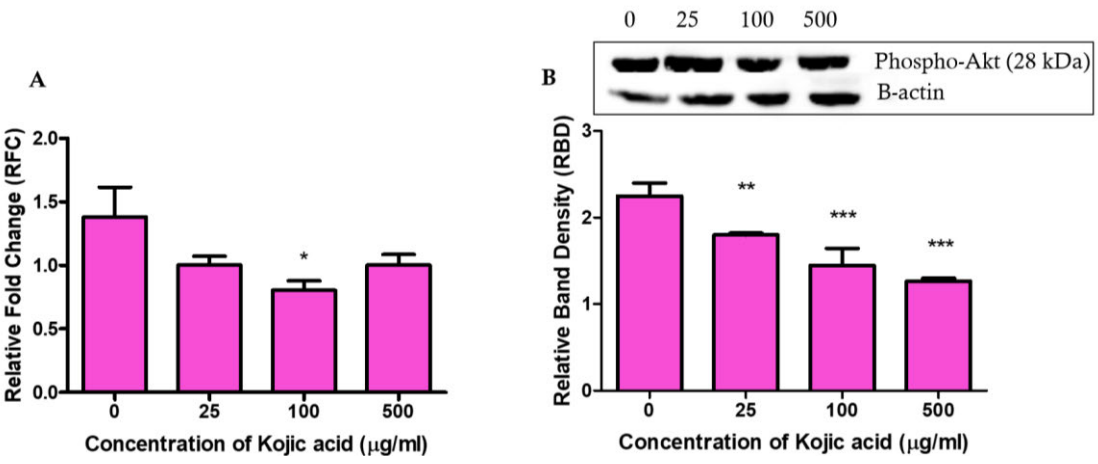
Statistical analysis

GraphPad Prism version 5.0 (GraphPad Prism Software Inc.) was used for all statistical analyses. One-way analysis of variance (ANOVA) with Tukey multiple comparison tests (95% CI) was used to analyse data sets. Unless otherwise indicated, the results were expressed as the mean ± standard deviation (SD) (n = 3). Statistical significance was considered at $p < 0.05$.

Results

Kojic acid altered the protein kinase B signalling pathway

The study investigated the effect of KA on total *Akt* gene and phospho-Akt protein expression. The results indicated a significant reduction in both total *Akt* expression and phospho-Akt expression (Figure 4.1A, $p = 0.0149$; Figure 4.1B, $p < 0.0001$) after KA treatment. There was also a decrease in phospho-GSK3β expression (Figure 4.1C, $p = 0.0004$) by KA. The results show that Akt signalling was downregulated after 24 h of exposure.



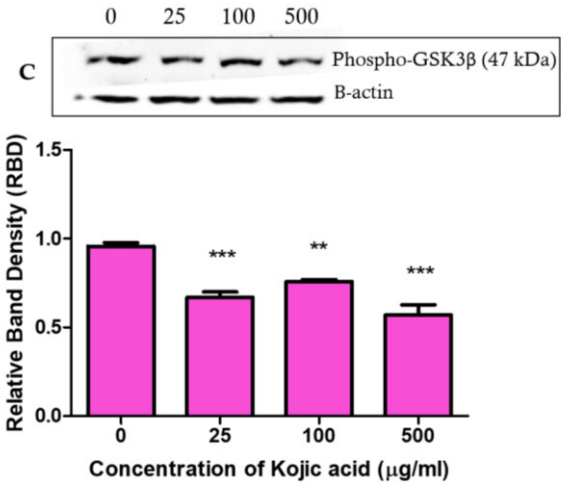


Figure 4.1: Kojic acid treatment resulted in alterations in Akt and GSK3β expression in SK-MEL-1 cells. Both the (A) total *Akt* gene expression and (B) phospho-Akt protein levels were significantly reduced. (C) There was a significant decline in phospho-GSK3β protein expression. The findings are presented as mean fold-change ± SD (n = 3) with ***p < 0.001, **p < 0.01, and *p < 0.05 denoting statistical significance.

Kojic acid decreased the expression of MAPK ERK1/2 and p38 signalling pathway

Inflammatory responses are influenced by the MAPK proteins (ERK1/2 and p38). After a 24 h treatment, the expression of ERK1/2 was significantly reduced across all concentrations of KA (Figure 4.2, p < 0.0001). Additionally, the expression of p38 was significantly lower than the control group (Figure 4.3, p < 0.0001).

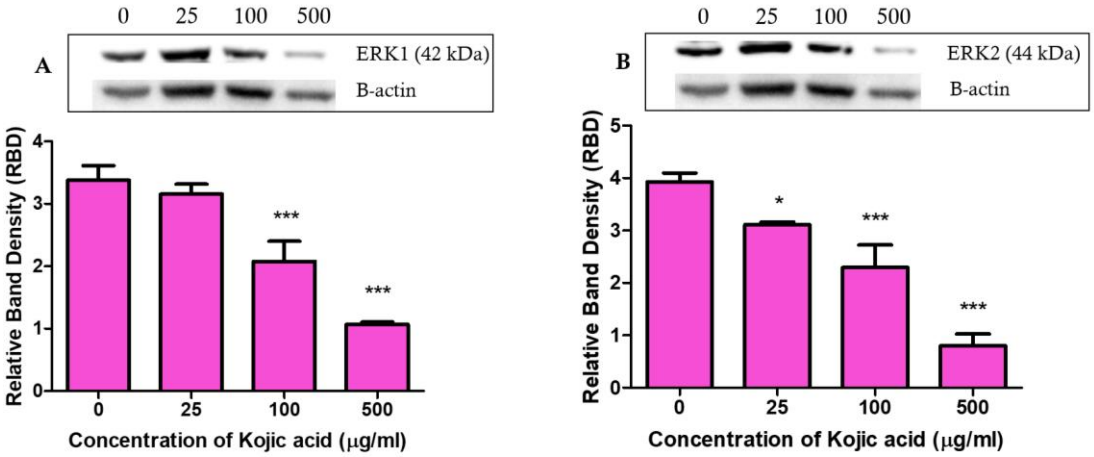


Figure 4.2: Kojic acid treatment suppressed ERK1/2 protein expression in SK-MEL-1 cells. Both the protein expression of (A) ERK1 & (B) ERK2 was significantly reduced. This reduction was particularly noteworthy at high treatment concentrations. The findings are presented as mean fold-change ± SD (n = 3) with ***p < 0.001 and *p < 0.05 denoting statistical significance.

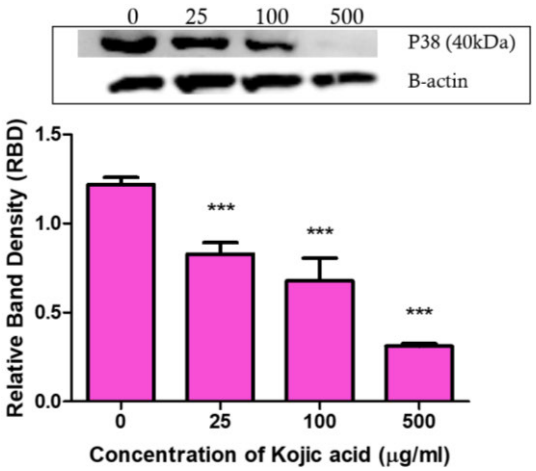


Figure 4.3: Kojic acid treatment altered p38 protein expression in SK-MEL-1 cells. There was a significant decrease in p38 protein expression. The findings are presented as mean fold-change ± SD (*n* = 3) with ***$p < 0.001$ denoting statistical significance.

Kojic acid exerted inflammatory effects on melanoma cells

The expression of inflammation markers was analysed due to KA's suppression of protein kinases (Akt, phospho-GSK3β, ERK1/2 and p38). Exposure to KA resulted in increased protein and gene expression of NFκB (Figure 4.4A, $p = 0.0007$; and Figure 4.4B, $p = 0.3648$) and *IκB* gene expression (Figure 4.4C, $p = 0.1758$) after 24 h exposure.

The activity of caspase 1 plays an integral part of the inflammasome. Results showed that lower concentrations of treatment led to an increase in caspase 1 and *NLRP3* expression after 24 h (Figure 4.5A, $p = 0.0168$; and Figure 4.5B, $p = 0.2993$). *IL-1β* and *IL-6* were increased specifically at the 100 µg/ml concentration (Figure 4.5C, $p = 0.0205$; and Figure 4.5D, $p = 0.472$), which increased NFκB expression.

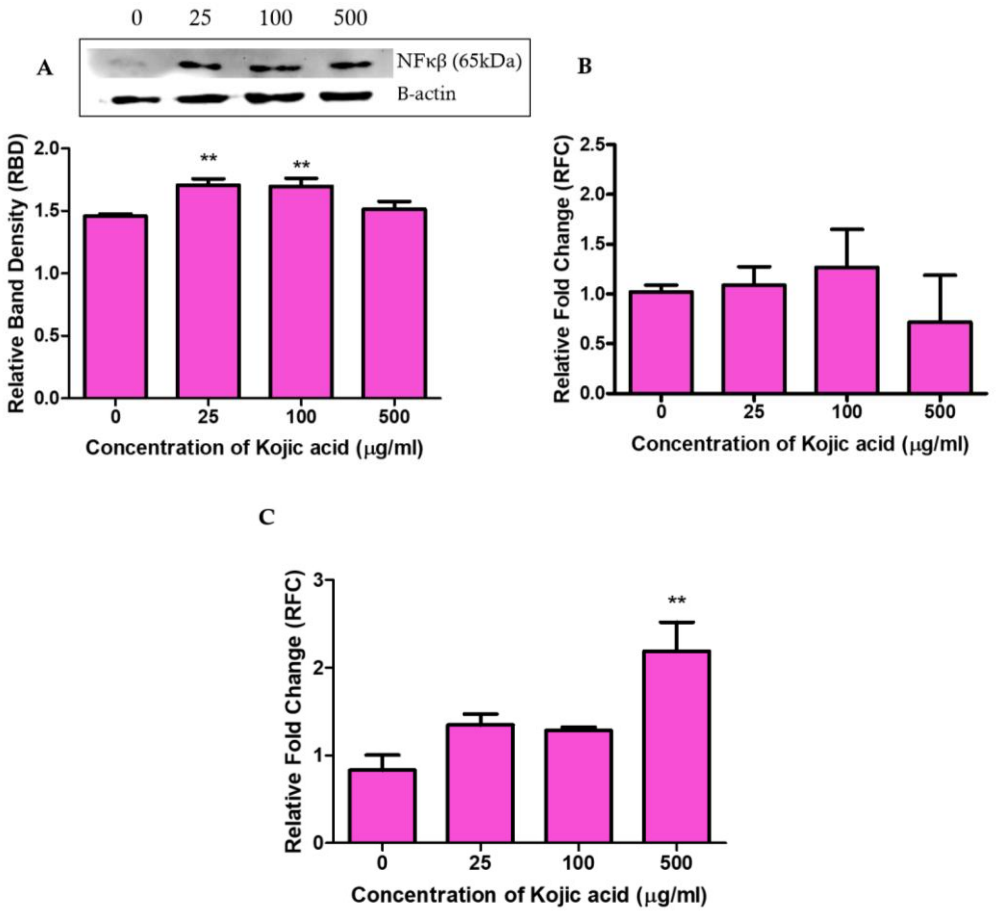
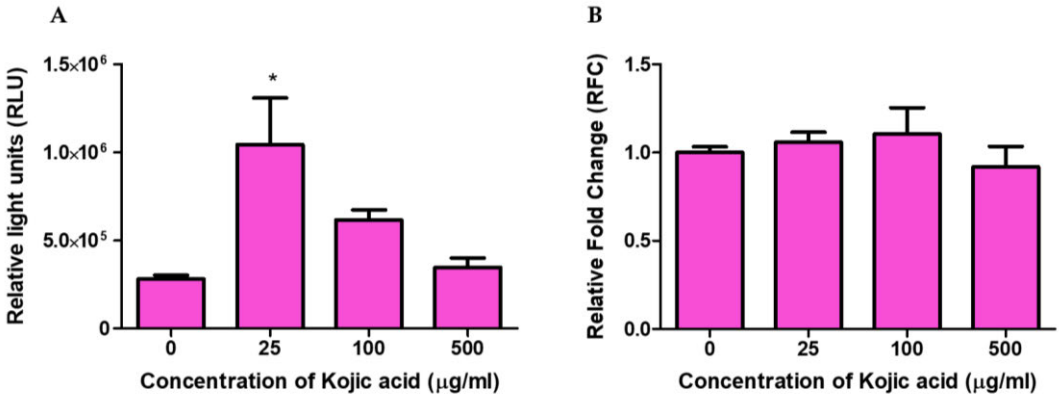


Figure 4.4: Kojic acid treatment increased NFκB expression in SK-MEL-1 cells. There was increased (A) NFκB protein and (B) *NFκB* gene expression. (C) Additionally, *IκB* gene expression was increased. The findings are presented as mean fold-change ± SD (n = 3) with $^{**}p < 0.01$ denoting statistical significance.



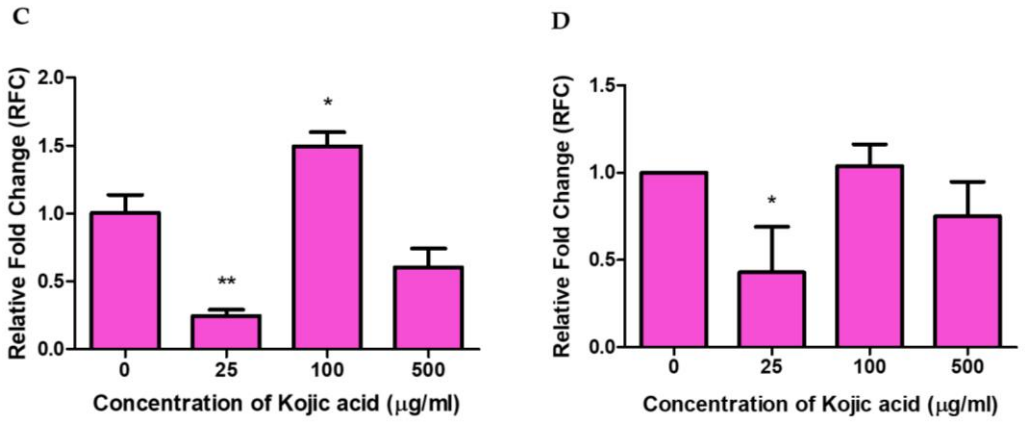


Figure 4.5: Kojic acid treatment altered the expression of key NLRP3 inflammasome mediators in SK-MEL-1 cells. (A) Intracellular caspase 1 and (B) *NLRP3* expression was increased. (C) *IL-1β* and (D) *IL-6* were increased significantly at the 100 μg/ml concentration. The findings are presented as mean fold-change ± SD ($n = 3$) with $^{**}p < 0.01$, and $^{*}p < 0.05$ denoting statistical significance.

Discussion

As the largest organ of the body, the skin is exposed to both extrinsic and intrinsic factors that can affect its health. The production of melanin, which gives colour to the skin, is the responsibility of melanocytes. This process involves a complex network of highly regulated signalling pathways. KA is known for its ability to interact with tyrosinase residues, which leads to a reduction in melanin production (Hussein-Al-Ali et al., 2014, Hashemi and Emami, 2015, Azami et al., 2017, Chen et al., 2019, Khezri et al., 2020). This function is enabled by the compound's capacity to chelate metals (Emami et al., 2007). In this study, we evaluated the impact of KA on protein kinases that play a crucial role in maintaining optimal cell functioning. Furthermore, we evaluated NFκB and key players in the NLRP3 inflammasome pathway. These protein kinases, such as Akt and MAPK, initiate inflammatory responses (Dong et al., 2002, Harikrishnan et al., 2018). Inflammation is important in activating the body's defence; however, excessive, and prolonged activation leads to adverse effects and must be highly controlled. This study investigated protein kinases and inflammatory mediators to assess KA's potential role in skin pathologies.

We used concentrations determined in our previous work to assess protein and gene expression of key signalling pathways and inflammatory mediators. We initially investigated the Akt/GSK3β signalling axis. Research into the relationship between Akt and NFκB revealed contrasting results. In monocytes, the decrease of PI_3K/Akt signalling leads to an increase in NFκB activation (Guha and Mackman, 2002); however, mouse macrophages and human endothelial cells show that PI_3K signalling activates NFκB responses (Ojaniemi et al., 2003, Li et al., 2003). We found that KA treatment resulted in the downregulation of *Akt* gene expression and phosphorylated Akt protein levels. A study carried out in mouse melanoma cells showed KA derivatives (1-60 μM) increased activated Akt expression which in

turn decreased melanin production. This contrasts with the results of previous studies in human melanoma cells (Chen et al., 2019). Furthermore, phospho-GSK3β was subsequently decreased (Figure 4.1). Under normal conditions, decreased Akt expression increases activated GSK3β expression. GSK3β post-translationally phosphorylates MITF and increases melanin synthesis (Fu et al., 2019). Following KA treatment, the decrease in phospho-GSK3β corresponds with KA's role in melanin synthesis inhibition (Lajis et al., 2012a, Zilles et al., 2022). The results depict KA inhibition of the Akt/GSK3β axis. Additionally, some studies state KA is a useful anti-ageing compound for reducing the appearance of wrinkles; however, little research assessed the biochemical pathways or associated proteins related to KA's anti-ageing potential. Inhibition of GSK3β activates fibroblasts and increases collagen release (Bergmann et al., 2011). More research studies, focussed on GSK3β inhibition will be beneficial to elucidate KA's anti-ageing potential.

Previous studies show that Akt pathways directly target MAPK signalling (Zimmermann and Moelling, 1999, Blum et al., 2001, Guha and Mackman, 2002, Galetic et al., 2003). In recent years, the cosmetic industry has targeted MAPK proteins due to their regulation of melanin synthesis in hyperpigmentation. Some studies have shown an inhibition of ERK signalling is due to Akt interaction (Rommel et al., 1999, Galetic et al., 2003, Schabbauer et al., 2004). We observed a significant dose-dependent decrease in the protein expression of ERK1/2 (Figure 4.2). We observed that the downregulation of Akt could have led to the downregulation of MAPK expression. GSK3 is a monomeric serine/threonine kinase that is regulated and decreased by ERK1/2 expression (Ding et al., 2005). The decrease in ERK1/2 expression could have potentially resulted in a decrease in GSK3β.

The p38 protein, a stress-activated protein kinase, functions in cellular processes such as cell proliferation, apoptosis, and inflammation (Cuadrado and Nebreda, 2010). Due to the SK-MEL-1 cell type and the dualistic nature of p38 in cell death and cell survival (Liu et al., 2014a), we conclude that a decrease in p38 expression potentially increased cell proliferation (Figure 4.3). This is consistent with the findings illustrating a relationship between p38 expression and tumours (Qiang et al., 2013). Previous research on liver cells demonstrated that KA directly increases p38 protein expression at high concentrations (Suthiram et al., 2023). Results showed that at low concentrations p38 expression is downregulated in melanoma cells.

The impact of downregulated protein kinase was further investigated by examining the gene and protein expression of inflammatory markers. It was discovered that the reduction in Akt resulted in an increase in NFκB gene and protein expression (Figure 4.4). GSK3 is a potent regulator of pro- and anti-inflammatory effects (Hoffmeister et al., 2020). GSK3 can act as a potent pro-inflammatory agent and inhibit these pathways by phosphorylating serine residues or terminating pro-inflammatory pathways (Hoffmeister et al., 2020). One such mechanism was illustrated by increased phosphorylation of the

NFκB inhibition site and decreasing phosphorylation of NFκB active sites. The results show an increase in NFκB with low *IκB* expression (Hoffmeister et al., 2020). Recent studies indicate that the proteins involved in MAPK signalling exhibit unique and varying forms of interaction with each other. MAPK p38 signalling initiates pro-inflammatory mediators in human and mouse models (Branger et al., 2002, Branger et al., 2003), whereas ERK1/2 suppresses the expression of pro-inflammatory mediators in keratinocytes (Pastore et al., 2005). This contrasts with HeLa cells where both ERK1/2 and p38 inhibit the expression of pro-inflammatory mediators including AP-1 and NFκB (Xiao et al., 2002). The results showed a concentration-dependent increase in inflammatory responses with a biphasic response to increasing treatment.

We observed that KA perturbed protein kinase signalling and increased cytokine release. Results showed elevated *IL-1β* and *IL-6* release at lower KA concentrations. The NLRP3 inflammasome was only activated at KA concentrations that was shown to display proliferation in our previous studies (Figure 4.5). There was a significant increase in the levels of intracellular caspase 1, which is necessary for the formation of the NLRP3 inflammasome. The efficient formation of inflammasomes leads to a subsequent increase in interleukin IL-1β and IL-6. Other studies have shown that p38 can regulate cytokines TNFα and IL-6 post-transcriptionally (Hochdörfer et al., 2013). Our research finds *IL-6* to display a hormetic response, similar to other inflammatory markers tested. However, unlike those markers, *IL-6* did not show a significant increase in activity at concentrations where the others did. This could be attributed to the decrease in p38 expression. KA has displayed anti-inflammatory properties by suppressing NFκB (Dung et al., 2014, Li et al., 2021, Khan et al., 2021), however, other studies show an increase in IL-6 expression (Choi et al., 2012, Montazeri et al., 2019). Additionally, some studies show that Akt suppresses NLRP3 activation and cytokine production (Zhao et al., 2020). Other kinases such as GSK3β act as initiators of inflammatory responses (Wang et al., 2020). NFκB plays a role in the priming step of NLRP3 inflammasome formation by increasing the expression of key components in the inflammasome and pro-IL-1β (Fetter et al., 2023).

Conclusion

In light of the results, we find that decreased protein kinases resulted in increased NFκB signalling and NLRP3 inflammasome formation and activation. Notably, these inflammatory responses were dependent on the concentrations used. This draws attention to the concentrations of KA present in cosmetic products. Inflammation is an exacerbator of many skin diseases therefore the regulation of KA concentrations is important specifically due to the observed biphasic responses. There are gaps in research regarding the toxicity and implications associated with the use of KA. We acknowledge that this study serves as a foundation for more exploratory research into the role of protein kinases and inflammation in the mechanism of skin lightening and anti-ageing. It would be beneficial to further

investigate the relationship between protein kinases and inflammatory mediators by assessing the role of microRNAs and regulation on the epigenetic level. Research focused on these pathways in this context will be beneficial for KA use in cosmetics.

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Authors contributions

Conceptualisation: KTS, TG, and AC. Methodology: KTS. Formal analysis and investigation: KTS. Writing- original draft: KTS. Review and editing: KTS, TG, and AC. Supervision: TG and AC.

Disclosure Statement

The authors declare no conflict of interest.

References

ASATI, V., MAHAPATRA, D. K. & BHARTI, S. K. 2016. PI3K/Akt/mTOR and Ras/Raf/MEK/ERK signaling pathways inhibitors as anticancer agents: Structural and pharmacological perspectives. *European journal of medicinal chemistry,* 109**,** 314-341.

AZAMI, F., TAZIKEH-LEMESKI, E. & MAHMOOD-JANLOU, M.-A. 2017. Kojic Acid Effect on the Inhibitory Potency of Tyrosinase. *Journal of Chemical Health Risks,* 7**,** 147-155. DOI: 10.22034/jchr.2017.544176.

BERGMANN, C., AKHMETSHINA, A., DEES, C., PALUMBO, K., ZERR, P., BEYER, C., ZWERINA, J., DISTLER, O., SCHETT, G. & DISTLER, J. H. W. 2011. Inhibition of glycogen synthase kinase 3β induces dermal fibrosis by activation of the canonical Wnt pathway. *Annals of the Rheumatic Diseases,* 70**,** 2191-2198. DOI: 10.1136/ard.2010.147140.

BLUM, S., ISSBRUKER, K., WILLUWEIT, A., HEHLGANS, S., LUCERNA, M., MECHTCHERIAKOVA, D., WALSH, K., VON DER AHE, D., HOFER, E. & CLAUSS, M. 2001. An inhibitory role of the phosphatidylinositol 3-kinase-signaling pathway in vascular endothelial growth factor-induced tissue factor expression. *Journal of Biological Chemistry,* 276**,** 33428-33434.

BRANGER, J., VAN DEN BLINK, B., WEIJER, S., GUPTA, A., VAN DEVENTER, S. J., HACK, C. E., PEPPELENBOSCH, M. P. & VAN DER POLL, T. 2003. Inhibition of coagulation, fibrinolysis, and endothelial cell activation by a p38 mitogen-activated protein kinase inhibitor during human endotoxemia. *Blood,* 101**,** 4446-4448.

BRANGER, J., VAN DEN BLINK, B., WEIJER, S., MADWED, J., BOS, C. L., GUPTA, A., YONG, C.-L., POLMAR, S. H., OLSZYNA, D. P. & HACK, C. E. 2002. Anti-inflammatory effects of

a p38 mitogen-activated protein kinase inhibitor during human endotoxemia. *The journal of immunology,* 168**,** 4070-4077.

CHAMCHEU, J. C., ROY, T., UDDIN, M. B., BANANG-MBEUMI, S., CHAMCHEU, R. N., WALKER, A. L., LIU, Y. Y. & HUANG, S. 2019. Role and Therapeutic Targeting of the PI3K/Akt/mTOR Signaling Pathway in Skin Cancer: A Review of Current Status and Future Trends on Natural and Synthetic Agents Therapy. *Cells,* 8. DOI: 10.3390/cells8080803.

CHEN, Y.-M., SU, W.-C., LI, C., SHI, Y., CHEN, Q.-X., ZHENG, J., TANG, D.-L., CHEN, S.-M. & WANG, Q. 2019. Anti-melanogenesis of novel kojic acid derivatives in B16F10 cells and zebrafish. *International Journal of Biological Macromolecules,* 123**,** 723-731. DOI: https://doi.org/10.1016/j.ijbiomac.2018.11.031.

CHOI, H., KIM, K., HAN, J., CHOI, H., JIN, S. H., LEE, E. K., SHIN, D. W., LEE, T. R., LEE, A.-Y. & NOH, M. 2012. Kojic acid-induced IL-6 production in human keratinocytes plays a role in its anti-melanogenic activity in skin. *Journal of Dermatological Science,* 66**,** 207-215. DOI: https://doi.org/10.1016/j.jdermsci.2012.03.002.

COLEMAN, M. L., MARSHALL, C. J. & OLSON, M. F. 2004. RAS and RHO GTPases in G1-phase cell-cycle regulation. *Nature reviews Molecular cell biology,* 5**,** 355-366.

CUADRADO, A. & NEBREDA, A. R. 2010. Mechanisms and functions of p38 MAPK signalling. *Biochemical journal,* 429**,** 403-417.

DÉRIJARD, B., RAINGEAUD, J., BARRETT, T., WU, I.-H., HAN, J., ULEVITCH, R. J. & DAVIS, R. J. 1995. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science,* 267**,** 682-685.

DING, Q., XIA, W., LIU, J.-C., YANG, J.-Y., LEE, D.-F., XIA, J., BARTHOLOMEUSZ, G., LI, Y., PAN, Y. & LI, Z. 2005. Erk associates with and primes GSK-3β for its inactivation resulting in upregulation of β-catenin. *Molecular cell,* 19**,** 159-170.

DONG, C., DAVIS, R. J. & FLAVELL, R. A. 2002. MAP kinases in the immune response. *Annual review of immunology,* 20**,** 55-72.

DOZA, Y. N., CUENDA, A., THOMAS, G. M., COHEN, P. & NEBREDA, A. R. 1995. Activation of the MAP kinase homologue RK requires the phosphorylation of Thr-180 and Tyr-182 and both residues are phosphorylated in chemically stressed KB cells. *FEBS letters,* 364**,** 223-228.

DUNG, T. T. M., KIM, S. C., YOO, B. C., SUNG, G.-H., YANG, W. S., KIM, H. G., PARK, J. G., RHEE, M. H., PARK, K. W., YOON, K., LEE, Y., HONG, S., KIM, J.-H. & CHO, J. Y. 2014. (5-Hydroxy-4-oxo-4H-pyran-2-yl)methyl 6-hydroxynaphthalene-2-carboxylate, a kojic acid derivative, inhibits inflammatory mediator production via the suppression of Syk/Src and NF-κB activation. *International Immunopharmacology,* 20**,** 37-45. DOI: https://doi.org/10.1016/j.intimp.2014.02.019.

ELLIOTT, E. I. & SUTTERWALA, F. S. 2015. Initiation and perpetuation of NLRP3 inflammasome activation and assembly. *Immunological Reviews,* 265**,** 35-52. DOI: https://doi.org/10.1111/imr.12286.

EMAMI, S., HOSSEINIMEHR, S. J., TAGHDISI, S. M. & AKHLAGHPOOR, S. 2007. Kojic acid and its manganese and zinc complexes as potential radioprotective agents. *Bioorganic & Medicinal Chemistry Letters,* 17**,** 45-48. DOI: https://doi.org/10.1016/j.bmcl.2006.09.097.

ESPINOSA-DIEZ, C., MIGUEL, V., MENNERICH, D., KIETZMANN, T., SÁNCHEZ-PÉREZ, P., CADENAS, S. & LAMAS, S. 2015. Antioxidant responses and cellular adjustments to oxidative stress. *Redox Biology,* 6**,** 183-197. DOI: https://doi.org/10.1016/j.redox.2015.07.008.

FANG, F., XIE, Z., QUAN, J., WEI, X., WANG, L. & YANG, L. 2020. Baicalin suppresses Propionibacterium acnes-induced skin inflammation by downregulating the NF-κB/MAPK signaling pathway and inhibiting activation of NLRP3 inflammasome. *Brazilian Journal of Medical and Biological Research,* 53.

FETTER, T., DE GRAAF, D. M., CLAUS, I. & WENZEL, J. 2023. Aberrant inflammasome activation as a driving force of human autoimmune skin disease. *Frontiers in Immunology,* 14. DOI: 10.3389/fimmu.2023.1190388.

FU, T., CHAI, B., SHI, Y., DANG, Y. & YE, X. 2019. Fargesin inhibits melanin synthesis in murine malignant and immortalized melanocytes by regulating PKA/CREB and P38/MAPK signaling pathways. *Journal of Dermatological Science,* 94**,** 213-219.

GALETIC, I., MAIRA, S.-M., ANDJELKOVIC, M. & HEMMINGS, B. A. 2003. Negative regulation of ERK and Elk by protein kinase B modulates c-Fos transcription. *Journal of Biological Chemistry,* 278**,** 4416-4423.

GUHA, M. & MACKMAN, N. 2002. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *Journal of Biological Chemistry,* 277**,** 32124-32132.

HARIKRISHNAN, H., JANTAN, I., HAQUE, M. A. & KUMOLOSASI, E. 2018. Anti-Inflammatory Effects of Hypophyllanthin and Niranthin Through Downregulation of NF-κB/MAPKs/PI3K-Akt Signaling Pathways. *Inflammation,* 41**,** 984-995. DOI: 10.1007/s10753-018-0752-4.

HASHEMI, S. M. & EMAMI, S. 2015. Kojic acid-derived tyrosinase inhibitors: synthesis and bioactivity. *Pharmaceutical and Biomedical Research,* 1**,** 1-17. DOI: 10.18869/acadpub.pbr.1.1.1.

HAYDEN, M. S. & GHOSH, S. 2012. NF-κB, the first quarter-century: remarkable progress and outstanding questions. *Genes & development,* 26**,** 203-234.

HOCHDÖRFER, T., TIEDJE, C., STUMPO, D. J., BLACKSHEAR, P. J., GAESTEL, M. & HUBER, M. 2013. LPS-induced production of TNF-α and IL-6 in mast cells is dependent on p38 but independent of TTP. *Cellular signalling,* 25**,** 1339-1347.

HOFFMEISTER, L., DIEKMANN, M., BRAND, K. & HUBER, R. 2020. GSK3: A Kinase Balancing Promotion and Resolution of Inflammation. *Cells,* 9**,** 820.

HUSSEIN-AL-ALI, S. H., EL ZOWALATY, M. E., HUSSEIN, M. Z., ISMAIL, M., DORNIANI, D. & WEBSTER, T. J. 2014. Novel kojic acid-polymer-based magnetic nanocomposites for medical applications. *International Journal of Nanomedicine,* 9**,** 351-362. DOI: 10.2147/ijn.S53847.

JEON, Y.-J., KIM, B.-H., KIM, S., OH, I., LEE, S., SHIN, J. & KIM, T.-Y. 2013. Rhododendrin ameliorates skin inflammation through inhibition of NF-κB, MAPK, and PI3K/Akt signaling. *European Journal of Pharmacology,* 714**,** 7-14. DOI: https://doi.org/10.1016/j.ejphar.2013.05.041.

KHAN, A., PARK, T. J., IKRAM, M., AHMAD, S., AHMAD, R., JO, M. G. & KIM, M. O. 2021. Antioxidative and Anti-inflammatory Effects of Kojic Acid in Aβ-Induced Mouse Model of Alzheimer's Disease. *Molecular Neurobiology,* 58**,** 5127-5140. DOI: 10.1007/s12035-021-02460-4.

KHEZRI, K., SAEEDI, M., MORTEZA-SEMNANI, K., AKBARI, J. & ROSTAMKALAEI, S. S. 2020. An emerging technology in lipid research for targeting hydrophilic drugs to the skin in the treatment of hyperpigmentation disorders: kojic acid-solid lipid nanoparticles. *Artificial Cells, Nanomedicine, and Biotechnology,* 48**,** 841-853. DOI: 10.1080/21691401.2020.1770271.

KIM, J.-H., HONG, S.-K., WU, P.-K., RICHARDS, A. L., JACKSON, W. T. & PARK, J.-I. 2014. Raf/MEK/ERK can regulate cellular levels of LC3B and SQSTM1/p62 at expression levels. *Experimental cell research,* 327**,** 340-352.

KONG, T., LIU, M., JI, B., BAI, B., CHENG, B. & WANG, C. 2019. Role of the Extracellular Signal-Regulated Kinase 1/2 Signaling Pathway in Ischemia-Reperfusion Injury. *Frontiers in Physiology,* 10. DOI: 10.3389/fphys.2019.01038.

LAJIS, A. F. B., HAMID, M. & ARIFF, A. B. 2012. Depigmenting effect of Kojic acid esters in hyperpigmented B16F1 melanoma cells. *Journal of Biomedicine & Biotechnology,* 2012**,** 1-9. DOI: 10.1155/2012/952452.

LI, T.-X., LIANG, J.-X., LIU, L.-L., SHI, F.-C., JIA, X.-W., LI, M.-H. & XU, C.-P. 2021. Novel kojic acid derivatives with anti-inflammatory effects from Aspergillus versicolor. *Fitoterapia,* 154**,** 105027. DOI: https://doi.org/10.1016/j.fitote.2021.105027.

LI, X., TUPPER, J. C., BANNERMAN, D. D., WINN, R. K., RHODES, C. J. & HARLAN, J. M. 2003. Phosphoinositide 3 kinase mediates Toll-like receptor 4-induced activation of NF-κB in endothelial cells. *Infection and immunity,* 71**,** 4414-4420.

LIU, L., REZVANI, H. R., BACK, J. H., HOSSEINI, M., TANG, X., ZHU, Y., MAHFOUF, W., RAAD, H., RAJI, G., ATHAR, M., KIM, A. L. & BICKERS, D. R. 2014. Inhibition of p38

MAPK Signaling Augments Skin Tumorigenesis via NOX2 Driven ROS Generation. *PLOS ONE,* 9**,** e97245. DOI: 10.1371/journal.pone.0097245.

LU, N. & MALEMUD, C. J. 2019. Extracellular Signal-Regulated Kinase: A Regulator of Cell Growth, Inflammation, Chondrocyte and Bone Cell Receptor-Mediated Gene Expression. *International Journal of Molecular Sciences,* 20. DOI: 10.3390/ijms20153792.

MONTAZERI, M., EMAMI, S., ASGARIAN-OMRAN, H., AZIZI, S., SHARIF, M., SARVI, S., REZAEI, F., SADEGHI, M., GOHARDEHI, S. & DARYANI, A. 2019. In vitro and in vivo evaluation of kojic acid against Toxoplasma gondii in experimental models of acute toxoplasmosis. *Experimental Parasitology,* 200**,** 7-12. DOI: https://doi.org/10.1016/j.exppara.2019.03.009.

OJANIEMI, M., GLUMOFF, V., HARJU, K., LILJEROOS, M., VUORI, K. & HALLMAN, M. 2003. Phosphatidylinositol 3-kinase is involved in Toll-like receptor 4-mediated cytokine expression in mouse macrophages. *European journal of immunology,* 33**,** 597-605.

OVERMEYER, J. H. & MALTESE, W. A. 2011. Death pathways triggered by activated Ras in cancer cells. *Frontiers in bioscience: a journal and virtual library,* 16**,** 1693.

PARK, Y. C., LEE, C. H., KANG, H. S., CHUNG, H. T. & DO KIM, H. 1997. Wortmannin, a specific inhibitor of phosphatidylinositol-3-kinase, enhances LPS-induced NO production from murine peritoneal macrophages. *Biochemical and biophysical research communications,* 240**,** 692-696.

PASTORE, S., MASCIA, F., MARIOTTI, F., DATTILO, C., MARIANI, V. & GIROLOMONI, G. 2005. ERK1/2 Regulates Epidermal Chemokine Expression and Skin Inflammation1. *The Journal of Immunology,* 174**,** 5047-5056. DOI: 10.4049/jimmunol.174.8.5047.

QI, S., XIN, Y., GUO, Y., DIAO, Y., KOU, X., LUO, L. & YIN, Z. 2012. Ampelopsin reduces endotoxic inflammation via repressing ROS-mediated activation of PI3K/Akt/NF-κB signaling pathways. *International immunopharmacology,* 12**,** 278-287.

QIANG, L., WU, C., MING, M., VIOLLET, B. & HE, Y.-Y. 2013. Autophagy controls p38 activation to promote cell survival under genotoxic stress. *Journal of Biological Chemistry,* 288**,** 1603-1611.

ROMMEL, C., CLARKE, B. A., ZIMMERMANN, S., NUNEZ, L., ROSSMAN, R., REID, K., MOELLING, K., YANCOPOULOS, G. D. & GLASS, D. J. 1999. Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science,* 286**,** 1738-1741.

ROY, T., BOATENG, S. T., UDDIN, M. B., BANANG-MBEUMI, S., YADAV, R. K., BOCK, C. R., FOLAHAN, J. T., SIWE-NOUNDOU, X., WALKER, A. L., KING, J. A., BUERGER, C., HUANG, S. & CHAMCHEU, J. C. 2023. The PI3K-Akt-mTOR and Associated Signaling Pathways as Molecular Drivers of Immune-Mediated Inflammatory Skin Diseases: Update on Therapeutic Strategy Using Natural and Synthetic Compounds. *Cells,* 12. DOI: 10.3390/cells12121671.

SCHABBAUER, G., TENCATI, M., PEDERSEN, B., PAWLINSKI, R. & MACKMAN, N. 2004. PI3K-Akt Pathway Suppresses Coagulation and Inflammation in Endotoxemic Mice. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 24**,** 1963-1969. DOI: doi:10.1161/01.ATV.0000143096.15099.ce.

SIVAPRASAD, U. & BASU, A. 2008. Inhibition of ERK attenuates autophagy and potentiates tumour necrosis factor-α-induced cell death in MCF-7 cells. *Journal of cellular and molecular medicine,* 12**,** 1265-1271.

SUTHIRAM, K. T., GHAZI, T., ABDUL, N. S. & CHUTURGOON, A. A. 2023. Kojic acid induces oxidative stress and anti-inflammatory effects in human hepatocellular carcinoma (HepG2) cells. *Toxicon,* 232**,** 107221. DOI: 10.1016/j.toxicon.2023.107221.

TAK, P. P. & FIRESTEIN, G. S. 2001. NF-κB: a key role in inflammatory diseases. *The Journal of Clinical Investigation,* 107**,** 7-11. DOI: 10.1172/JCI11830.

WANG, S., SU, X., XU, L., CHANG, C., YAO, Y., KOMAL, S., CHA, X., ZANG, M., OUYANG, X., ZHANG, L. & HAN, S. 2020. Glycogen synthase kinase-3β inhibition alleviates activation of the NLRP3 inflammasome in myocardial infarction. *Journal of Molecular and Cellular Cardiology,* 149**,** 82-94. DOI: https://doi.org/10.1016/j.yjmcc.2020.09.009.

XIAO, Y. Q., MALCOLM, K., WORTHEN, G. S., GARDAI, S., SCHIEMANN, W. P., FADOK, V. A., BRATTON, D. L. & HENSON, P. M. 2002. Cross-talk between ERK and p38 MAPK mediates selective suppression of pro-inflammatory cytokines by transforming growth factor-beta. *Journal of Biological Chemistry,* 277**,** 14884-93. DOI: 10.1074/jbc.M111718200.

YIN, L., NIU, C., LIAO, L.-X., DOU, J., HABASI, M. & AISA, H. A. 2017. An isoxazole chalcone derivative enhances melanogenesis in B16 melanoma cells via the Akt/GSK3β/β-catenin signaling pathways. *Molecules,* 22**,** 2077.

YOON, J. Y., KWON, H. H., MIN, S. U., THIBOUTOT, D. M. & SUH, D. H. 2013. Epigallocatechin-3-Gallate Improves Acne in Humans by Modulating Intracellular Molecular Targets and Inhibiting P. acnes. *Journal of Investigative Dermatology,* 133**,** 429-440. DOI: 10.1038/jid.2012.292.

ZALEŚNA, I., HARTMAN, M. L. & CZYŻ, M. 2016. BRAF mutation in progression and therapy of melanoma, papillary thyroid carcinoma and colorectal adenocarcinoma. *Advances in Hygiene and Experimental Medicine,* 70**,** 471-488.

ZANG, D., NIU, C. & AISA, H. A. 2019. Amine derivatives of furocoumarin induce melanogenesis by activating Akt/GSK-3β/β-catenin signal pathway. *Drug Design, Development and Therapy***,** 623-632.

ZHAO, W., SHI, C. S., HARRISON, K., HWANG, I. Y., NABAR, N. R., WANG, M. & KEHRL, J. H. 2020. AKT Regulates NLRP3 Inflammasome Activation by Phosphorylating NLRP3 Serine 5. *Journal of Immunology,* 205**,** 2255-2264. DOI: 10.4049/jimmunol.2000649.

ZILLES, J. C., DOS SANTOS, F. L., KULKAMP-GUERREIRO, I. C. & CONTRI, R. V. 2022. Biological activities and safety data of kojic acid and its derivatives: A review. *Experimental Dermatology,* 31**,** 1500-1521. DOI: https://doi.org/10.1111/exd.14662.

ZIMMERMANN, S. & MOELLING, K. 1999. Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science,* 286**,** 1741-1744.

CHAPTER 5

5.1. CONCLUSION

Skin-lightening products are a regular component of skin care routines in Asia and Africa. Most skincare products use KA due to its lightening properties. Therefore, it is important to determine KA's toxicity to ensure safe use. Following KA exposure, an assessment of key markers involved in the maintenance of good skin health helps to effectively treat and prevent disease. In recent years, the increase of KA uses due to other skin lighteners proving unfavourable brings to light the lack of research in terms of the biochemical effects of its use and its role in the ageing of the skin. KA has varying classifications in terms of being classified as a mycotoxin. The IARC states that KA is a group 3 carcinogen. This implies that there is no carcinogenic risk associated with its use. Most studies carried out on mice and rat models showed thyroid hyperplasia and skin sensitisation (Fujimoto et al., 1999, Tamura et al., 1999, Mitsumori et al., 1999, Higa et al., 2002, Tamura et al., 2006). Studies have also investigated KA's potential as an antioxidant, anti-inflammatory agent, and tyrosinase chelator.

There are gaps in research regarding the toxicity and implications associated with the use of KA. The study serves as a preliminary study demonstrating altered expression of key regulatory pathways (Nrf2 and NFκB) required for cellular responses and signalling following KA exposure.

KA induced protein oxidation in melanoma cells. Nrf2 is responsible for regulating the levels of ROS in the body, ensuring that they remain at non-toxic concentrations. This study highlights KA's ability to regulate antioxidant responses within melanoma cells. In light of the results obtained, we find that KA initiated antioxidant response proteins and altered mitochondrial dynamics. On the one hand, increased Nrf2 expression protects cells from oxidative stress damage; on the other hand, prolonged Nrf2 expression can be harmful by accelerating the development of cancer (Jaramillo and Zhang, 2013). Although it is unfavourable that cancer cells can 'hijack' Nrf2 to facilitate cell survival, Nrf2 activation is necessary to maintain low oxidative conditions and inhibit the progression of inflammatory disease (Kim and Keum, 2016). Upregulation of Nrf2 is important in skin metabolism due to its role in the protection of cells during melanogenesis (Gęgotek and Skrzydlewska, 2015). Additionally, elevated Nrf-2 expression has been shown to play a role in melanin inhibition (Shin et al., 2014). Dysregulation in Nrf2 activation can lead to cancer or cell death. Nrf2 was insufficient to counteract the accumulation of ROS resulting in protein damage.

Nrf2 binds to response elements found in DNA to facilitate cellular defence responses (Taguchi et al., 2011). Nrf2 is crucial for the regulation of more than one hundred genes such as LON protease and NFκB (Heiss et al., 2001, Pinti et al., 2011). LON protease is ATP-dependent and acts as a chaperone

for oxidised proteins. Altering LON protease expression results in protein carbonyl accumulation. KA post-transcriptionally regulated LON protease expression by increasing Nrf2 protein expression. Increased oxidative stress will initiate mitochondrial machinery to assists in maintaining homeostasis. Sirtuin 3 is a crucial element of mitochondrial homeostasis machinery. Sirtuins are NAD^+ dependent. The decrease in Sirtuin 3 protein expression suggests that NAD^+ levels could potentially be disrupted. Studies illustrate KA's chelation ability and interaction with the electron transport chain (Murakami, 1962, Nurchi et al., 2011a, Kim et al., 2013a). KA can target key complexes in the electron transport chain thereby affecting NAD^+ levels. This suggests that KA was mitotoxic to melanoma cells following acute exposure.

In response to oxidative conditions, KA increased NFκB expression in melanoma cells. Intracellular signaling pathways play a role in mediating the cytoprotection activity of Nrf2 (Kim and Keum, 2016). Considering KA interacts with residues present in enzymes, as seen in tyrosinase inhibition, protein kinase (MAPK and Akt) signalling was assessed. Oxidative stress is also an activator of cell signalling pathways. There was a suppression of MAPK proteins (p38 and ERK1/2) as well as Akt signalling. KA downregulated protein kinase expression in melanoma cells. Lastly, the activation of inflammation was independent of protein kinases. This suggests that oxidative conditions (lipid peroxidation and protein carbonyls) stimulated downstream inflammatory pathways. The increase in key activators of the NLRP3 inflammasome resulted in the release of IL-1β and IL-6 causing inflammation (Figure 5.1). In conclusion, KA activated Nrf2 and NFκB expression which impacted downstream protein kinase targets. This is promising for future research related to skin-related therapies. Elevated Nrf2 expression and the subsequent regulation of downstream signalling could potentially contribute to KA's efficient skin lightening. The study assessed a range of KA concentrations to determine cytotoxicity in melanoma cells, whilst some concentrations displayed unfavourable effects. The concentrations that displayed beneficial effects could be utilised for future work to assess other related pathways that have not been studied as yet.

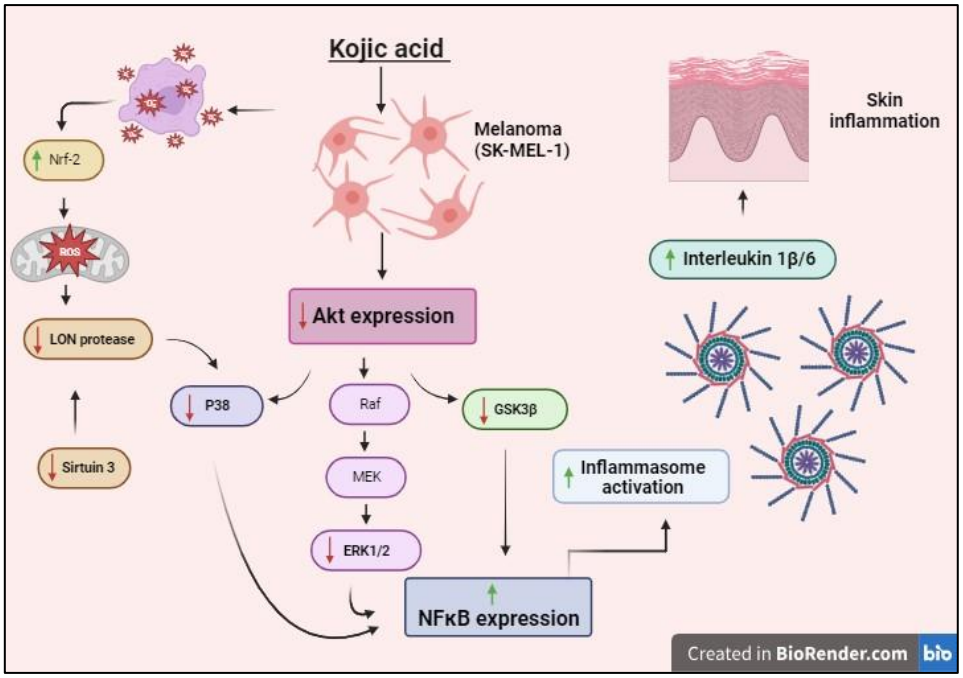

Figure 5.1: The effect of kojic acid on mitochondrial homeostasis markers, protein kinases and inflammation in SK-MEL-1 melanoma cells

5.2. LIMITATIONS AND RECOMMENDATIONS

The study found that acute KA exposure results in the oxidation of proteins and initiation of inflammatory responses, however, other studies found that prolonged exposure at differing concentrations leads to antioxidant and anti-inflammatory effects (Mohammadpour et al., 2013, Lee et al., 2019a, Khan et al., 2021, Li et al., 2021). Due to the use of a cancerous cell line, a primary cell line can be used to verify results as well as prolonged exposure to KA (greater than 24 h). The current preliminary study was *in vitro* SK-MEL-1 cells which evaluated KA's ability to mediate antioxidant responses. The SK-MEL-1 cell line was suitable due to its role in melanin production. However, an *in vitro* study does not accurately simulate the interactions between different cell lines in multicellular organisms. Therefore, the study can be replicated in *in vivo* research, followed by clinical trials in humans to determine the safety of KA use. This research bears importance as it evaluates, Nrf2, a key regulator of cell function and homeostasis. Many drug therapies targeting Nrf2 have been used in skin disease treatment. Understanding the role of KA in enhancing Nrf2 expression can aid in safe compound use.

References

FUJIMOTO, N., ONODERA, H., MITSUMORI, K., TAMURA, T., MARUYAMA, S. & ITO, A. 1999. Changes in thyroid function during development of thyroid hyperplasia induced by kojic acid in F344 rats. *Carcinogenesis,* 20**,** 1567-1572.

GĘGOTEK, A. & SKRZYDLEWSKA, E. 2015. The role of transcription factor Nrf2 in skin cells metabolism. *Archives of Dermatological Research,* 307**,** 385-96. DOI: 10.1007/s00403-015-1554-2.

HEISS, E., HERHAUS, C., KLIMO, K., BARTSCH, H. & GERHÄUSER, C. 2001. Nuclear factor κB is a molecular target for sulforaphane-mediated anti-inflammatory mechanisms. *Journal of Biological Chemistry,* 276**,** 32008-32015.

HIGA, Y., OHKUBO, A., KITAJIMA, S., MORIYASU, M. & KARIYA, K. 2002. Effects of kojic acid on thyroidal functions in rats by single-dose administration and in cultured rat thyroid cells (FRTL-5 cells). *The Journal of Toxicological Sciences,* 27**,** 423-431.

JARAMILLO, M. C. & ZHANG, D. D. 2013. The emerging role of the Nrf2-Keap1 signaling pathway in cancer. *Genes and Development,* 27**,** 2179-91. DOI: 10.1101/gad.225680.113.

KHAN, A., PARK, T. J., IKRAM, M., AHMAD, S., AHMAD, R., JO, M. G. & KIM, M. O. 2021. Antioxidative and Anti-inflammatory Effects of Kojic Acid in Aβ-Induced Mouse Model of Alzheimer's Disease. *Molecular Neurobiology,* 58**,** 5127-5140. DOI: 10.1007/s12035-021-02460-4.

KIM, J. & KEUM, Y.-S. 2016. NRF2, a Key Regulator of Antioxidants with Two Faces towards Cancer. *Oxidative Medicine and Cellular Longevity,* 2016**,** 2746457. DOI: 10.1155/2016/2746457.

KIM, J. H., CAMPBELL, B. C., CHAN, K. L., MAHONEY, N. & HAFF, R. P. 2013. Synergism of Antifungal Activity between Mitochondrial Respiration Inhibitors and Kojic Acid. *Molecules,* 18**,** 1564-1581.

LEE, M., RHO, H. S. & CHOI, K. 2019. Anti-inflammatory Effects of a P-coumaric Acid and Kojic Acid Derivative in LPS-stimulated RAW264.7 Macrophage Cells. *Biotechnology and Bioprocess Engineering,* 24**,** 653-657. DOI: 10.1007/s12257-018-0492-1.

LI, T.-X., LIANG, J.-X., LIU, L.-L., SHI, F.-C., JIA, X.-W., LI, M.-H. & XU, C.-P. 2021. Novel kojic acid derivatives with anti-inflammatory effects from Aspergillus versicolor. *Fitoterapia,* 154**,** 105027. DOI: https://doi.org/10.1016/j.fitote.2021.105027.

MITSUMORI, K., ONODERA, H., TAKAHASHI, M., FUNAKOSHI, T., TAMURA, T., YASUHARA, K., TAKEGAWA, K. & TAKAHASHI, M. 1999. Promoting effects of kojic acid due to serum TSH elevation resulting from reduced serum thyroid hormone levels on development of thyroid proliferative lesions in rats initiated with N-bis (2-hydroxypropyl) nitrosamine. *Carcinogenesis,* 20**,** 173-176.

MOHAMMADPOUR, M., BEHJATI, M., SADEGHI, A. & FASSIHI, A. 2013. Wound healing by topical application of antioxidant iron chelators: kojic acid and deferiprone. *International Wound Journal,* 10**,** 260-264. DOI: https://doi.org/10.1111/j.1742-481X.2012.00971.x.

MURAKAMI, Y. 1962. Complexing behaviour of kojic acid with metal ions—II: Fe(III) chelates. *Journal of Inorganic and Nuclear Chemistry,* 24**,** 679-688. DOI: https://doi.org/10.1016/0022-1902(62)80086-4.

NURCHI, V. M., LACHOWICZ, J. I., CRISPONI, G., MURGIA, S., ARCA, M., PINTUS, A., GANS, P., NICLOS-GUTIERREZ, J., DOMÍNGUEZ-MARTÍN, A., CASTINEIRAS, A., REMELLI, M., SZEWCZUK, Z. & LIS, T. 2011. Kojic acid derivatives as powerful chelators for iron(III) and aluminium(III). *Dalton Transactions,* 40**,** 5984-98. DOI: 10.1039/c1dt00005e.

PINTI, M., GIBELLINI, L., DE BIASI, S., NASI, M., ROAT, E., O'CONNOR, J. E. & COSSARIZZA, A. 2011. Functional characterization of the promoter of the human Lon protease gene. *Mitochondrion,* 11**,** 200-6. DOI: 10.1016/j.mito.2010.09.010.

SHIN, J.-M., KIM, M. Y., SOHN, K.-C., JUNG, S.-Y., LEE, H.-E., LIM, J. W., KIM, S., LEE, Y.-H., IM, M., SEO, Y.-J., KIM, C. D., LEE, J.-H., LEE, Y. & YOON, T.-J. 2014. Nrf2 Negatively Regulates Melanogenesis by Modulating PI3K/Akt Signaling. *PLOS ONE,* 9**,** e96035. DOI: 10.1371/journal.pone.0096035.

TAGUCHI, K., MOTOHASHI, H. & YAMAMOTO, M. 2011. Molecular mechanisms of the Keap1–Nrf2 pathway in stress response and cancer evolution. *Genes to cells,* 16**,** 123-140.

TAMURA, T., MITSUMORI, K., ONODERA, H., FUJIMOTO, N., YASUHARA, K., TAKEGAWA, K. & TAKAHASHI, M. 1999. Inhibition of thyroid iodine uptake and organification in rats treated with kojic acid. *Toxicological Sciences,* 47**,** 170-175. DOI: 10.1093/toxsci/47.2.170.

TAMURA, T., MITSUMORI, K., TOTSUKA, Y., WAKABAYASHI, K., KIDO, R., KASAI, H., NASU, M. & HIROSE, M. 2006. Absence of in vivo genotoxic potential and tumor initiation activity of kojic acid in the rat thyroid. *Toxicology*, 222**,** 213-224.

Addendum

Addendum A:

The following study, “Kojic acid induces oxidative stress and anti-inflammatory effects in human hepatocellular carcinoma (HepG2) cells” provided insight into potential adverse effects of KA and set the foundation for the current study.

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Kojic acid induces oxidative stress and anti-inflammatory effects in human hepatocellular carcinoma (HepG2) cells

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ABSTRACT

The cosmetic industry makes extensive use of kojic acid (KA); however, the toxicity of KA in humans is not well known. By monitoring oxidative stress, mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NFκB) signalling in human hepatoma (HepG2) cells after a 24 h exposure, this study aimed to identify the toxicity of KA. KA toxicity [4.22, 8.02 and 12.67 mM] was assessed using mitochondrial output, antioxidant responses, macromolecule damage, MAPK signalling, inflammation, and cell death markers, using spectrophotometry, luminometry, Western blot and qPCR. Apoptosis was confirmed by reduced cell viability and increased caspases −9 ($p < 0.0001$), −8 ($p = 0.0003$), and −3/7 ($p < 0.0001$) activities at 4.22 mM and 8.02 mM. LDH leakage was present at 12.67 mM, providing significant evidence of necrosis. Malondialdehyde (MDA) levels significantly increased at 4.22 mM ($p < 0.0001$). There was an increase of phosphorylated nuclear factor erythroid-2 factor-2 (p-Nrf2) at 4.22 mM and 8.02 mM, whilst at 12.67 mM decreased p-Nrf2 ($p < 0.0001$) was observed. KA increased p38 expression ($p = 0.0011$). The findings point to significant suppression of the NFκB inflammatory pathway at 8.02 mM ($p < 0.0001$). This study showed that KA initiated MAPK signalling due to oxidative stress and suppressed inflammation. HepG2 cells showed minimal toxicity to KA.

1. Introduction

In the pharmaceutical, cosmetic, and food industries, 5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one, commonly referred to as kojic acid (KA), is a widely used hydrophilic chelating agent and skin lightener (Azami et al., 2017). The lightning effect is achieved by inhibiting tyrosinase activity, which reduces melanin production in melanocytes (Choi et al., 2012; Zilles et al., 2022). The U.S. Food and Drug Administration (FDA) classifies *A. oryzae* (koji) as generally regarded as safe (GRAS), despite the fact that its safety has not been properly investigated (Hamajima et al., 2016). The FDA has not authorized KA for use in medicinal products for skin-lightning purposes (Belsito et al., 2009).

More than 500 metabolic processes are carried out by the liver. As a result of the liver's role in blood detoxification and drug metabolism, it is vulnerable to toxicity (Singh et al., 2011). KA toxicity studies assessing the impact on human liver cells are limited. Known attributes of KA include antibacterial activity (Nurunnabi et al., 2018), antifungal activity (Owolabi et al., 2020), antioxidant (Niwa and Akamatsu, 1991; Khan et al., 2021) and anti-inflammatory properties (Moon et al., 2001; Li et al., 2021; Khan et al., 2021).

One of the target organs of oxidative stress is the liver. Oxidative stress was found to mediate the progression of liver disease (Sánchez-valle et al., 2012). An imbalance between free radicals and antioxidants leads to oxidative stress. The molecular oxygen can react to produce substances such as hydrogen peroxide (H_2O_2), water (H_2O), and superoxide anions ($O_2^{\cdot-}$). The equilibrium required for the efficient functioning of biological systems can be disrupted when oxidants exceed antioxidant levels (Ott et al., 2007). Macromolecules that are exposed to excessive reactive oxygen species (ROS) are prone to lipid peroxidation and can develop protein carbonyls and DNA adducts (Wiesmüller et al., 2002; Cooke et al., 2003; Dalle-donne et al., 2003; Ayala et al., 2014).

In response to oxidative stress, nuclear factor erythroid-2 related factor-2 (Nrf2) stimulates the transcription of antioxidant genes, superoxide-dismutase 2 (SOD2) and catalase (CAT). Due to this role, Nrf2 is known as the master regulator of antioxidant responses. Nrf2 induces phase 2 responses for cellular defence mechanisms. KEAP-1, a repressor that contains zinc coupled to reactive cysteine thiols, must dissociate from Nrf2 for this process to proceed. These antioxidant enzymes play key roles in the alleviation of oxidative stress (Basak et al., 2017). SOD2 transforms $O_2^{\cdot-}$ into hydrogen peroxide (H_2O_2), which is

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then broken down by CAT. Dysfunction of antioxidant responses has been linked to several illnesses (Liguori et al., 2018).

Tyrosinase activity is inhibited by KA; therefore, the mitogen-activated protein kinase (MAPK) pathway may be a possible target. Toxic insults brought on by ROS can activate MAPK signalling for the initiation of cell death (Kim et al., 2014). Specific stress-activated protein kinases (SAPK), namely p38 and c-Jun-N-terminal kinase (JNK) can be impacted by the amount of oxidative stress that cells experience. Transcriptional factors are phosphorylated by MAPK proteins, which also affect biological processes such as cell death and survival. Although Nrf2 activation has been linked to the MAPK pathway, the specific process by which regulation occurs is still unknown. Studies conducted thus far have favoured the indirect phosphorylation of Nrf2 as the focus of MAPK pathway regulation (Sun et al., 2009). Prior research has not examined Nrf2 and MAPK signalling in HepG2 liver cells after KA treatment.

Inflammatory pathway activation occurs as a result of immune responses to oxidative stress and is a contributing factor to several diseases. NFκB and IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) are found in the cytoplasm; phosphorylated NFκB enters the nucleus and initiates transcription of pro-inflammatory cytokines, while activated IκBα is released for ubiquitin-dependent proteasome degradation (Yang et al., 2012).

MicroRNAs (miRNAs) are a class of post-transcriptional repressors of gene expression (Reinhart et al., 2000). MiRNAs mediate gene regulation by binding to target messenger RNA (mRNA) at the 3' untranslated region (Dexheimer and Cochella, 2020). MiRNAs are important regulators of oxidative stress, immunological response, inflammation, and cell death. Some miRNAs involved in these functions include miRNA-155, miRNA-29a and miRNA-29b. MiRNA-155 is involved in repressing NFκB and MAPK signalling (Wang et al., 2015). MiRNA-29a expression is inhibited by NFκB and targets pro-apoptotic mediators such as bcl-2-like protein 4 (Bax), poly(ADP-ribose) polymerase (PARP), phospho-Fas-associated protein with death domain (phospho-FADD), cleaved caspase-8, and caspase-3 (Tiao et al., 2014). MiRNA-29b regulates oxidative stress by decreasing cell viability and increasing apoptosis initiators (Hou et al., 2017).

The intensity and duration of oxidative stress dictate the extent of damage and the onset of cell death. Due to its ability to eliminate damaged cells, apoptosis is essential for the preservation of cellular homeostasis. Through intrinsic and extrinsic apoptotic pathways, caspases initiate (caspases-8 and -9) and execute (caspase-3/7) apoptosis (Elmore, 2007).

The effects of KA on oxidative stress, MAPK signalling and inflammation have been studied in animal and human models, but liver damage is still largely unexplored. This study examines the impact of KA on oxidative stress by assessing macromolecule damage (lipid peroxidation, 8-OHdG and protein carbonyls concentrations) and antioxidant responses (Nrf2, CAT and GPx expression). Furthermore, we assessed the MAPK (p38 and JNK 1/2) signalling pathway, and NFκB inflammatory responses in the human liver (HepG2) cell line. The findings of this study shed light on the role of KA in liver toxicity.

2. Materials and methods

2.1. Materials

The HepG2 cell line was purchased from the American Type Culture Collection (ATCC; Johannesburg, South Africa). Lonza Biotechnology (Basel, Switzerland) was used to obtain cell culture supplies. Kits for luminometry were purchased from Promega (Madison, WI, USA). Western Blot reagents were purchased from Bio-Rad (California, United States). Unless otherwise specified, every additional reagent and consumable was bought from Merck (Darmstadt, Germany).

2.2. Cell culture and treatment

Complete culture medium (CCM: Eagle's Minimum Essentials Medium supplemented with 1% penicillin-streptomycin-fungizone, 1% L-glutamine and 10% foetal calf serum) was used to cultivate HepG2 cells in 25 cm^3 cell culture flasks. A 37 °C humidified incubator with a steady flow of 5% CO_2 was used to cultivate the cells. The stock solution of KA (20 mg/ml) was prepared in 0.1 M phosphate-buffered saline (PBS). The cells were allowed to reach 90% confluency before KA treatment [0, 4.22, 8.02, and 12.67 mM]. For all assays, the results obtained following KA treatment were compared to the untreated control.

2.3. Methyl thiazol tetrazolium (MTT) assay

To ascertain the impact of KA on HepG2 cell viability, the MTT assay was performed. In a 96-well microtiter plate, HepG2 cells (1.5×10^6) were seeded and incubated overnight before being treated with KA at various doses (0–12.67 mM) for 24 h at 37 °C, 5% CO_2. CCM (100 μl) and MTT salt solution [20 μl; 5 mg/ml in 0.1 M PBS] was added to the cells and incubated (37 °C, 4 h). To dissolve the formazan crystals, DMSO (100 μl/well) was added, and the plate was incubated for 1 h at 37 °C. A microplate reader (Biotek μQuant Plate reader, Winooski, VT, USA) was used to measure the absorbance at 570 nm. The reference wavelength was 690 nm. GraphPad Prism V5.0 was used to analyse the obtained absorbances and the half-maximum inhibitory concentration (IC_{50}) was determined.

2.4. Crystal violet assay

The crystal violet assay was conducted according to (Feoktistova et al., 2016). HepG2 cells were attached to a 96-well microtiter plate and treated with different KA concentrations (0–12.67 mM) for 24 h at 37 °C and 5% CO_2. After the cells had been incubated, dH_2O was added to wash them, and 0.5% crystal violet dye (50 μl) was then added. On a bench shaker, the plates were incubated for 20 min at a rate of 20 oscillations per min. Cells were washed 4 consecutive times in dH_2O and air-dried overnight at room temperature (RT). The plate was then incubated at RT for 20 min with 200 μl of methanol added to each well. A microplate reader (Biotek μQuant Plate reader, Winooski, VT, USA) was used to measure the absorbance at a wavelength of 570 nm. Cell viability was assessed, relative to the control.

2.5. Activity of caspases −8, −9, and −3/7

By using luminometry, the impact of KA on caspase −3/7, −8, and −9 activity in HepG2 cells was evaluated. Following treatment with KA for 24 h, HepG2 cells (20,000 cells/well) were seeded in an opaque 96-well microtiter plate along with 25 μl of the Caspase Glo® −9, −8, and −3/7 reagents and the plate was incubated in the dark for 30 min at RT. On a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA), luminescence was measured. The results were presented as relative light units (RLU).

2.6. Lactate dehydrogenase (LDH) assay

The effect of KA on the integrity of cell membranes was assessed using the LDH cytotoxicity detection kit (Roche, Mannheim, Germany). After HepG2 cells had been exposed to KA for 24 h, supernatants (100 μl) were transferred in triplicate into a 96-well microtiter plate. After adding 100 μl of the INT/sodium lactate dye solution and the diaphorase/NAD+ catalyst to each well, the plate was incubated for 20 min at RT in the dark. Using a spectrophotometer (Biotek μQuant Plate reader, Winooski, VT, USA), optical density was measured at 500 nm. Results were shown as mean optical density.

2.7. ATP quantification assay

The ATP Cell Titre Glo® luminometry assay kit was used to quantify ATP levels as previously described (Abdul et al., 2016). In an opaque 96-well microtiter plate, 20,000 HepG2 cells were seeded in each well and the cells were then incubated for 30 min at RT with 20 µl of ATP Cell Titre Glo® reagent (Promega, Madison, USA). The Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA) was used to measure luminescence. Luminescence was quantified as relative light units (RLU) and is proportional to ATP levels.

2.8. Lipid peroxidation assay

Lipid peroxidation produces compounds called thiobarbituric acid reactive substances (TBARS). Since reactive oxygen species (ROS) have a short half-life, it is challenging to directly quantify them; as a result, TBARS analyses the by-product malondialdehyde (MDA), which is produced during lipid peroxidation and is a sign of oxidative stress. Briefly, 200 µl of supernatants were added to test tubes along with 0.1 M TBA/BHT solution (400 µl), 2% H_3PO_4 (200 µl), and 7% H_3PO_4 (200 µl). Additionally, a positive control containing 1% MDA (1 µl) and a negative control containing 3 mM HCl (400 µl) were prepared. After vortexing each sample, 200 µl of 1 M HCl was added to bring the pH to 1.5. Before adding butanol (1500 µl), samples were heated (100 °C, 15 min) and allowed to cool to RT. The samples were vortexed for 30 s and then left to separate into two phases. The butanol phase (100 µl) was added in triplicate to a 96-well microtiter plate and the absorbance was measured using a Biotek µQuant spectrophotometer (Winooski, VT, USA) at 532 nm with a reference wavelength of 600 nm. MDA concentration (mM) was calculated using the absorption coefficient (156 mM^{-1}).

2.9. Protein carbonyl assay

Crude protein was isolated from HepG2 cells using 200 µl cell lysis solution (Tris-Cl, 1% Triton × 100, 10% glycerol and 50 mmol/l NaCl). The proteins were standardised to 1.5 mg/ml. The standardised protein (200 µl) was added to DNPH (800 µl) at RT for 1 h. A blank consisting of the standardised protein (200 µl) and 2.5 M HCl (800 µl) was also prepared. During the 1 h incubation, the solution was vortexed at every 15 min interval. After incubation, each sample was precipitated with 1 ml of 20% Trichloroacetic Acid (TCA) on ice, vortexed, and centrifuged (2000 g, 10 min, RT). Each sample's pellet was washed twice with 1 ml of ethanol-ethyl acetate (1:1) before being dissolved in 500 µl of 6 mol/l guanidine hydrochloride. To remove any insoluble material, samples were incubated (37 °C, 10 min) and centrifuged (2000×g, 10 min) at RT. The supernatant (100 µl) was added in triplicate into a 96-well microtiter plate (100 µl) and the absorbance was measured at 370 nm with a spectrophotometer. The average absorbance obtained from the blank was subtracted from the sample absorbance to determine the corrected absorbance. The protein carbonyl concentration was obtained by dividing the corrected absorbance calculated by the 2,4-Dinitrophenol's (DNP's) extinction coefficient (22,000 $l.M^{-1}.cm^{-1}$). The results were expressed in nanomoles per milligram units.

2.10. Protein expression analysis

Western Blot was used to determine the protein expression of JNK, p38, Nrf2, p-ser40-Nrf2 and CAT. Using CytoBuster™ reagent (200 µl) supplemented with protease and phosphatase inhibitors, proteins were isolated from control and KA-treated HepG2 cells. Before being mechanically lysed, samples were incubated for 30 min on ice. The samples underwent a 10,000×g centrifugation at 4 °C for 10 min. The bicinchoninic acid assay was used to quantify the proteins which were subsequently standardised to 1.5 mg/ml. Laemmli buffer (50 µl; dH_2O, 0.5 M Tris-Cl (6.8), bromophenol blue, β-mercaptoethanol, glycerol and 10% SDS) was added to the samples and boiled (100 °C) for 5 min.

Table 1
The annealing temperatures of primer sequences for gene expression.

Gene	Sequence	Annealing temperature (°C)
NFκB (p65)	Forward (5'-ACCAACAACAACCCCTTCCA-3') Reverse (5'-GTAGTCCCCACGCTGCTCTT-3')	60
IκBα	Forward (5'-CACTCCATCCTGAAGGCTACCAAC-3') Reverse (5'-CACACTTCAACAGGAGTGACACCAG-3')	64
GPx	Forward (5'-GACTACACCCAGATGAACGAGC-3') Reverse (5'-AATCCCCAGCAGTGGAATAAGG-3')	58
GAPDH	Forward (5'-TCCACCACCCTGTTGCTGTA-3') Reverse (5'-ACCACAGTCCATGCCATCAC-3')	Same as gene of interest

Thereafter, samples were electrophoresed in an SDS-PAGE gel (4% stacking gel, 7.5% resolving gel) at 150V (Bio-Rad compact power supply) for 1.5 h. The separated proteins were transferred onto nitrocellulose membranes for 30 min with a Trans-Blot Turbo System (Bio-Rad). Blocking of the membranes was performed using 5% BSA for 1 h with gentle rotation. The membranes were incubated with primary antibody (1:5000), anti-JNK (SAB 4200176, Sigma-Aldrich), anti-Nrf2 (12721S, Cell Signalling), anti-p-ser40-Nrf2 (AB76026, Abcam), anti-catalase (C0979, Sigma-Aldrich), anti-p38 (M8177, Sigma-Aldrich) for 1 h (RT) and overnight at 4 °C. At 10 min intervals, the membranes were washed five times with TTBS (150 mM NaCl, 3 mM KCl, 25 mM Tris, 0.05% Tween 20, dH2O). Horseradish peroxidase-conjugated secondary antibody (goat anti-mouse (7076, Cell Signalling Technology) and goat anti-rabbit (7074, Cell Signalling Technology), 1:5000) was added to the membranes and incubated at RT for 1 h. Protein bands were visualised using the Clarity Western ECL detection reagent (Bio-Rad) and the Bio-Rad ChemiDoc™ XRS+ System. Images were analysed using ImageLab Software™ v6.0 (Bio-Rad).

Membranes were quenched in 5% hydrogen peroxide (30 min, 37 °C). Membranes were blocked with 5% BSA and incubated in HRP-conjugated antibody for β-actin (Abcam) as a housekeeping protein to normalise expression. The results were presented as relative band density (RBD), which was calculated from the ratio of the RBD of the target protein and the respective β-actin.

2.11. Gene expression analysis

RNA was isolated from KA-treated HepG2 cells as per (Chuturgoon et al., 2014). The RNA was quantified using the Nanodrop 2000 spectrophotometer (Thermo-Fischer Scientific, Waltham, USA) and standardised to 1000 ng/µl cDNA was synthesized from standardised RNA using the Maxima™ H Minus cDNA synthesis kit (Thermo-Fisher Scientific). The conditions for the thermocycler were as follows: 25 °C for 10 min, 50 °C for 15 min, and 85 °C for 5 min.

The gene expression of *GPx*, *IκBα*, and *NFκB(p65)* was investigated using the PowerUp™ SYBR™ Green Master Mix (Thermo-Fisher Scientific) together with the respective primers (Table 1). Reaction volumes were prepared as follows: SYBR green (5 µl), forward primer (1 µl), reverse primer (1 µl), nuclease-free water (2 µl) and cDNA template (1 µl). Each reaction was carried out in triplicate.

The CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) was used to amplify the samples. Cycling conditions were as follows: initial denaturation (8 min, 95 °C) followed by 40 cycles of denaturation (15 s, 95 °C), annealing (40 s, temperature - Table 1) and extension (30 s, 72 °C). The relative change in gene expression was assessed using the method ($2^{-\Delta\Delta Ct}$) described by (Livak and Schmittgen, 2001). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a house-keeping gene, was used to normalise gene expression.

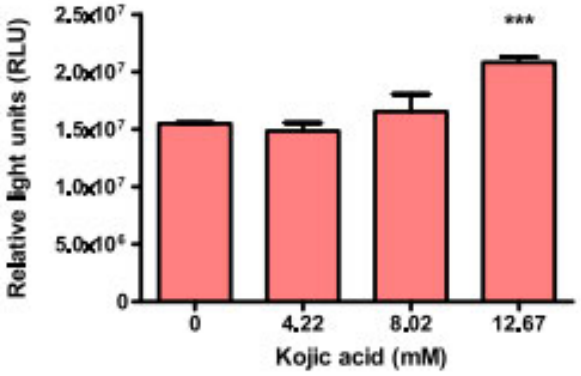

Fig. 1. Intracellular ATP levels following KA treatment in HepG2 cells for 24 h. The data were represented as mean ± SD ($n = 3$).

2.12. DNA isolation and 8-OHdG enzyme-linked immunosorbent assay (ELISA)

Cells were treated with KA for 24 h. The treatment was removed and washed with PBS three times. After removing the PBS, the cell lysis solution was added and incubated for 15 min at RT. A cell scraper was used to mechanically scrape the cells until a homogeneous solution was formed. Potassium acetate (600 µl) was added, mixed with a vortex, and inverted for 8 min in a fume hood. The solution was vortexed and centrifuged for 5 min at 13,000 rpm. The supernatant was decanted, and isopropanol (600 µl) was added. The solution was invert mixed and subsequently centrifuged for 5 min. A white pellet was collected, and care was taken to avoid disturbing the DNA. The isopropanol was removed and to the pellet, 300 µl of ethanol was added. The solution was vortexed and centrifuged at 13,000 rpm for 5 min. The ethanol was decanted and inverted on a clean, absorbent paper towel to dry the pellet for 15 min. The pellet was resuspended in DNA hydration solution (TE buffer), vortexed and placed on a dry block heater at 65 °C for 15 min. Once heated, the tubes were allowed to cool for 15 min. Crude DNA concentration was quantified using the Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, USA). All samples were standardised to 100 ng/ml.

For the ELISA, the preparation of 8-OHdG standards was carried out by completing a serial dilution as per the manufacturer's instructions. Duplicate additions of standards (50 µl), and isolated DNA samples (50 µl) were transferred to the 96-well microtiter plate. Antibody diluent was used to dilute the anti-8-OHdG, and 50 µl was then transferred to the respective wells except for the blank. For 1 h, the plates were covered and incubated at RT. Using wash buffer (300 µl), the wells were washed five times and then inverted to drain any liquid. A working solution comprised of the anti-mouse antibody diluted in HRP diluent as per manufacturers' instruction. Each well received 100 µl of the working solution, which was then incubated (1 h, RT). The well was aspirated and washed five times (300 µl, RT) and inverted to remove liquid. Each well received 100 µl of TMB substrate solution before being incubated for 15 min at RT. Stop solution (100 µl) was pipetted into each well after incubation. On a spectrophotometer, the absorbance was measured at 450 nm.

2.13. Statistical analysis

GraphPad Prism V5.0 Software (GraphPad Prism Software Inc.) was used to analyse the data. To display the data and establish statistical significance, one-way analysis of variance (ANOVA) and Tukey multiple comparison tests (95% CI) were utilized. The data was represented as mean ± standard deviation (SD) ($n = 3$), unless otherwise stated.

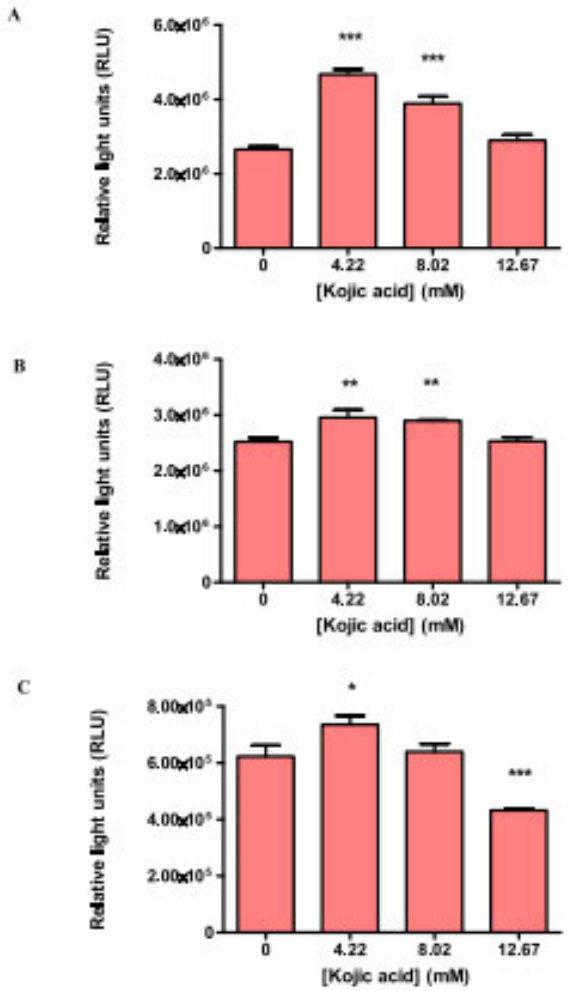

Fig. 2. The effect of KA treatment effects on (A) caspase 9, (B) caspase 8, and (C) caspase 3/7 activity in HepG2 cells. The data was represented as mean ± SD ($n = 3$).

Significant data obtained a p-value below 0.05.

3. Results

3.1. KA decreased cell viability and mitochondrial output in HepG2 cells

To determine cell viability and mitochondrial output, MTT and crystal violet assays were performed. Mitochondrial output was decreased following a 24 h KA treatment. HepG2 cell viability was lowered by KA in a dose-dependent manner, with an IC_{50} of 8.02 mM (Figure 9A, Supplementary). Additionally, a significant loss in cell viability was seen at 4.22 mM and 12.67 mM (Figure 9B, $p < 0.0001$, Supplementary); these concentrations were used for all future assays. The HepG2 cells were treated with varying concentrations of KA ranging from 0 to 12.67 mM for a period of 48 h at 37 °C (Figure. 10). The IC_{50} obtained was 6.3 mM. The IC_{50} was within the range of the 24 h treatment hence no further treatments at 48 h were carried out. ATP levels were significantly elevated at the higher KA concentrations (12.67 mM) relative to the control (Fig. 1, $p < 0.0001$).

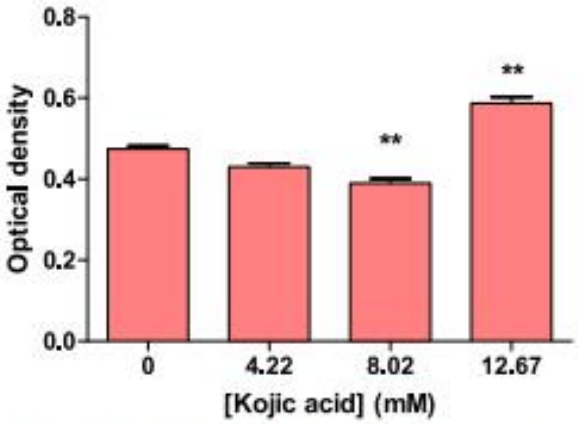

Fig. 3. KA treatment affects LDH leakage in HepG2 cells. The data were represented as mean ± SD ($n = 3$).

3.2. Kojic acid induced cell death in HepG2 cells

Caspase −9, −8, and −3/7 activities as well as LDH leakage were evaluated to detect cell death in HepG2 cells. There was an increase in caspase 9 ($p < 0.0001$, Fig. 2A) and caspase 8 ($p = 0.0003$, Fig. 2B) activity compared to the control. Caspase 3/7 activity was significantly increased by the 4.22 mM concentration of KA but decreased by the 12.67 mM treatment ($p < 0.0001$, Fig. 2C). At 4.22 mM and 8.02 mM kA LDH levels was decreased whereas, at 12.67 mM of KA significant membrane damage was experienced ($p < 0.0001$, Fig. 3). The decrease in caspase 3/7 activity and the elevation in LDH leakage strongly support necrosis as the mode of cell death.

3.3. Kojic acid induced oxidative stress in HepG2 cells

Macromolecules were evaluated in HepG2 cells after KA treatment to validate cell death induction due to stress responses. In HepG2 cells, KA treatment changed the cell's antioxidant status. Production of extracellular MDA, a by-product of lipid peroxidation, was significantly increased by KA ($p < 0.0001$, Fig. 4A) relative to the control. Lipid peroxidation indicators, such as 4-HNE, can create DNA adducts, which result in DNA breakage. Although elevated lipid peroxidation markers were detected, it was found that KA did not significantly elevate 8-OHdG levels ($p = 0.0025$, Fig. 4B). Protein carbonyls were assessed in KA-treated cells and were significantly decreased compared to the control ($p < 0.0001$, Fig. 4C).

3.4. Kojic acid decreased antioxidant response proteins

At higher KA concentrations (12.67 mM), the expression of antioxidant response proteins, Nrf2 and p-Nrf2, was significantly downregulated in HepG2 cells. In response to oxidative stress, Nrf2 ($p = 0.0002$, Fig. 5A) and p-Nrf2 ($p < 0.0001$, Fig. 5B) expression was increased by lower concentrations (4.22 mM and 8.02 mM) of KA.

Following KA treatment, CAT, a component in the detoxification of peroxides, was downregulated ($p = 0.0002$, Fig. 5C). Utilizing qPCR, the expression of *GPx* was examined. *GPx* levels were increased at 4.22 mM (1.51 fold) and 8.02 mM (1.07 fold); however, at 12.67 mM (1.02 fold), *GPx* expression was unchanged relative to the control ($p = 0.0120$, Fig. 5D).

3.5. Kojic acid induced an immune response by MAPK proteins

Stress-activated protein kinases (SAPKs), JNK 1/2 and p38, are involved in inflammation, cell regulation, and apoptosis. MAPK proteins were evaluated using Western blot to ascertain the immunological response to KA. JNK 1/2 expression was decreased by KA relative to the control (Fig. 6A ($p = 0.0039$) & 6B ($p < 0.0001$)). The protein expression of p38 was significantly upregulated at concentrations 4.22 and 8.02 mM of KA but remained unchanged at 12.67 mM ($p = 0.0011$, Fig. 6C).

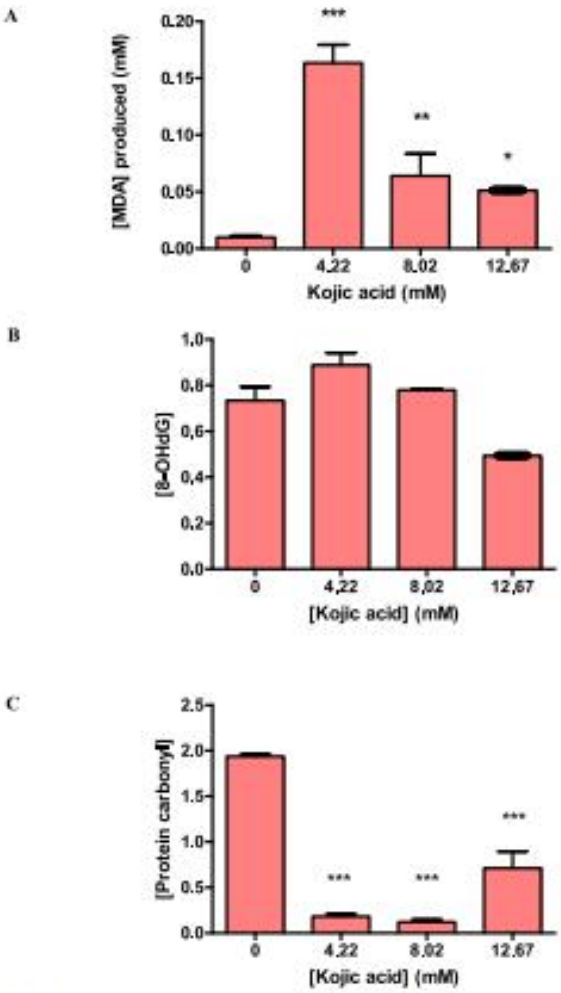

Fig. 4. The effect of KA on cellular oxidation in HepG2 cells. KA elevated (A) extracellular MDA concentration and decreased the concentrations of (B) 8-OHdG and (C) protein carbonyls after 24 h treatment. The data was represented as mean ± SD ($n = 3$).

3.6. Kojic acid induced an anti-inflammatory response in HepG2 cells

Inflammatory signals are essential for cell function in the presence of toxic insults. To verify KA's anti-inflammatory properties, qPCR was used. *NFκB* gene expression was decreased at 4.22 mM (0.82 fold), and 8.02 mM (0.24 fold); however, *NFκB* expression at 12.67 mM (1.02 fold) was unchanged relative to the control ($p = 0.0029$, Fig. 7A). It is notable that at 8.02 mM, *NFκB* expression significantly decreased. Likewise, at 8.02 mM, *IκB* expression was decreased. *IκB* expression was upregulated relative to the control at 4.22 and 12.67 mM ($p < 0.0001$, Fig. 7B).

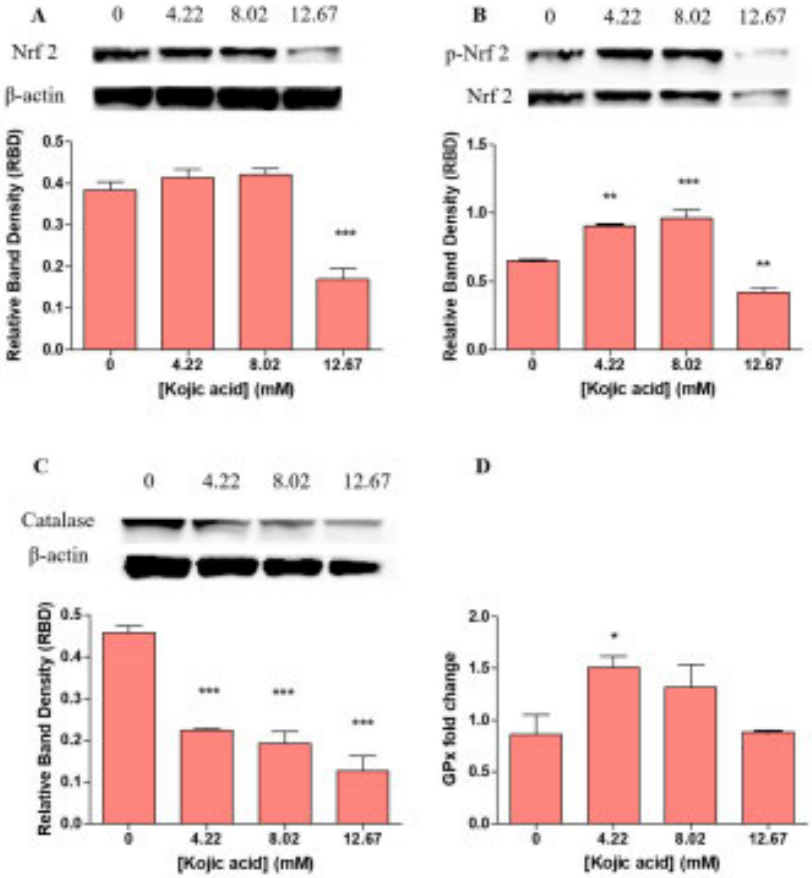

Fig. 5. The effect of KA on Nrf2 (A), p-Nrf2 (B), CAT (C), and *GPx* in HepG2 cells. The data was represented as mean ± SD ($n = 3$).

3.7. Kojic acid increased the expression of miRNAs involved in oxidative stress and immune response in HepG2 cells

Following KA treatment, the *miRNA-29a* expression was significantly upregulated at 12.67 mM ($p = 0.0002$, Fig. 8A). *MiRNA-29b* expression was increased following KA-treatment ($p = 0.0009$, Fig. 8B). *MiRNA-155* was significantly upregulated at 4.22 mM ($p = 0.0018$, Fig. 8C).

4. Discussion

KA is found in a variety of foods and cosmetics. The toxicity of KA on both human and animal skin cells has come to light due to its uses in skin lightening (Kim et al., 2003; Lajis et al., 2012). It is beneficial to understand better the effects of KA on organs that are responsible for toxin metabolism and excretion. Using liver HepG2 cells as a model, the current study assessed how KA affected important biochemical processes namely, oxidative stress and inflammation.

The effect of KA on human liver cells was assessed using cell viability assays. KA significantly reduced cell viability in a dose-dependent manner, particularly so at high concentrations (Fig. 9A & B, Supplementary). Similarly, in a previous study using low KA concentrations [0 – 400 μM], the viability of cardiomyocytes remained unchanged following treatment (Chaudhari et al., 2018). Cell viability was not significantly reduced in the MTT assay; however, significant cell death was evident in the crystal violet assay. No significant mitochondrial dysregulation (MTT assay) due to KA exposure was observed, with the exception of 12.67 mM. KA inhibits cell proliferation and reduces tumour growth (Chen et al., 2013). It is well known that weak acids, like KA, can inhibit cell growth by reducing the pH of the cell and affecting the mitochondria (Stratford and Anslow, 1998; Kundukad et al., 2020; Kim et al., 2013).

The efficiency of cellular metabolism in the mitochondria depends on the availability of ATP. The mitochondria produce ATP through the process of oxidative phosphorylation. (Ott et al., 2007). The high metabolic activity of the liver depends on a large density of mitochondria. A decrease in cell viability raises the possibility of mitochondrial dysfunction. Since most ROS are produced during mitochondrial respiration, the mitochondria are vulnerable to injury (Ott et al., 2007). KA significantly upregulated ATP production at high concentrations (Fig. 1). The rise in ATP is regarded as a "stress signal", which mediates downstream signalling. Necrosis is a form of cell death that is ATP-dependent; however, cancer cells have the capacity to use LDH to aerobically metabolize ATP. This could account for the increase of ATP at high concentrations of KA. Notably, ATP levels were unaffected at low KA concentrations and decreased following a 400 μM kA treatment in cardiomyocytes. In the same study, LDH leakage was also undetected at low concentrations (0 – 400 μM) (Chaudhari et al., 2018).

LDH leakage/membrane integrity and apoptosis activating caspases −9, −8, and −3/7 were measured to confirm cell death. Caspase −8, −9, and −3/7 expression levels were increased, which was indicative of successful apoptosis induction (Fig. 2). In agreement with previous studies, KA induced apoptosis by stimulating copper-induced apoptotic agents in HeLa cells as a response to oxidative stress (Chen et al., 2013). KA derivatives displayed copper-chelating properties that initiated

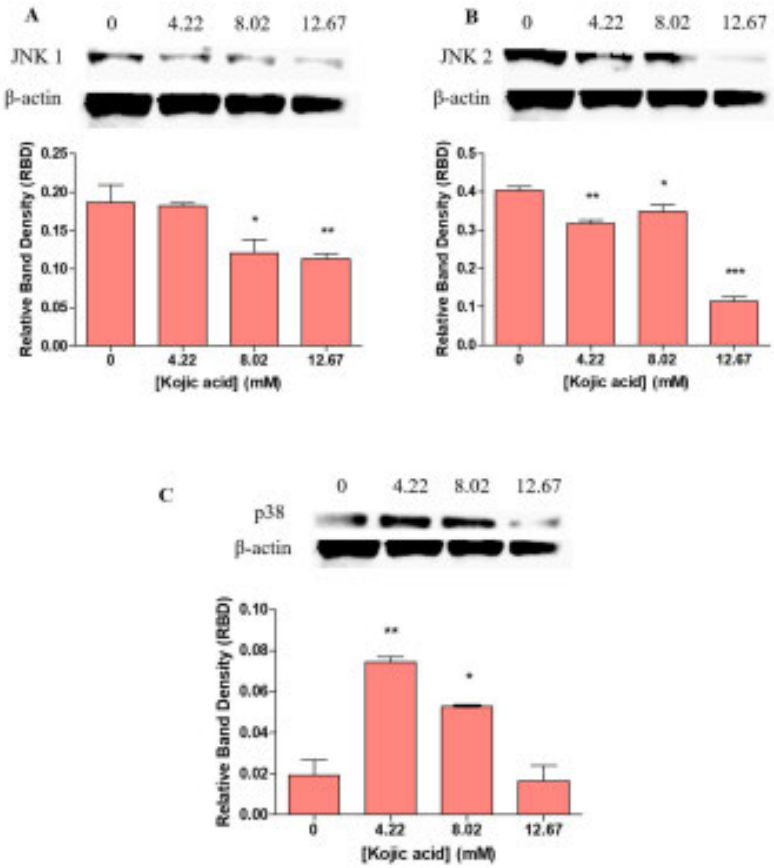

Fig. 6. The effect of KA on immune response by MAPK signalling A) JNK 1, B) JNK 2, C) p38. The data were represented as mean ± SD ($n = 3$).

apoptosis in HepG2 cells (Oncul et al., 2019). However, the present investigation has found that, at lower concentrations, KA itself has enhanced caspase −3/7, −8, and −9 activity, leading to cell apoptosis. Additionally, it was noted that LDH levels significantly decreased, indicating that there was no leakage or disruption to cellular membranes (Fig. 3). Interestingly, caspase −8 and −9 and executioner caspase −3/7 activity was unchanged at high concentrations. However, LDH levels was increased, suggesting that high concentrations of KA can damage cellular membranes leading to necrosis. A dose-dependent decrease was observed in Fig. 3 for 12.67 mM. Due to reduced levels of toxicity, HepG2 cells experience controlled cell death (apoptosis) at lower KA concentrations. Both the initiator and executioner caspases were activated. However, KA is more harmful to cells at greater concentrations, resulting in membrane damage. Necrosis may have occurred based on the high LDH levels observed at high concentrations.

MDA generation in HepG2 cells was enhanced by KA, indicating the presence of oxidative stress (Fig. 4A). The results illustrated elevated MDA production at low concentrations but decreased MDA at higher concentrations. This adaptive response by cells is referred to as a hormetic response and could account for the lack of a dose-dependent response. Hormesis is a term used in toxicology to refer to any process in a cell that exhibits a biphasic response to exposure to increasing amounts of a toxin/compound usually with a low dose stimulation and high dose inhibition (Mattson, 2008). In the liver of iron-loaded Wistar rats, KA significantly increased MDA levels and did not exhibit any protective effects attributable to KA's chelating activity (Kotyzova et al., 2004). Toxicity studies discovered that KA's capacity to create ROS enhances the phagocytic activity in mouse peritoneal cells, resulting in cell injury (Rodrigues et al., 2011). *In vitro* KA studies, on the other hand, were discovered to have antioxidant properties in leukocytes by scavenging ROS (Niwa and Akamatsu, 1991).

The pH change affects the nucleic acid structure, reaction rates, protein stability, and enzyme performance (Kundukad et al., 2020). The level of protein and DNA damage was evaluated due to KA's weak acidic properties. DNA is crucial for the transport of information, therefore mutations can cause diseases such as cancer (Yin et al., 1995). Studies on KA's capacity to develop tumours in *in vivo* and *in vitro* models have produced contradictory results. However, research has drawn attention to KA's anti-carcinogenic properties as a positive attribute. It has been determined that DNA damage can contribute to the development of cancer. The most common DNA lesion, 8-OHdG was investigated. The data indicate a marginal rise in the number of oxidative lesions, although the variation was statistically insignificant (Fig. 4B). Rat thyroid 8-OHdG oxidative adducts were not significantly enhanced, according to *in vivo* genotoxic studies. Further examination was done on the colon, liver and stomach to corroborate *in vitro* findings, and it revealed that there was no significant rise in the number of oxidative lesions *in vivo* which is consistent with the published *in vitro* findings (Higa et al., 2007; Tamura et al., 2006). A decrease of 8-OHdG levels at the highest treatment concentration correlated with KA's potential anti-carcinogenic properties. Protein carbonyl concentrations were investigated to determine KA's ability to oxidise proteins. A significant

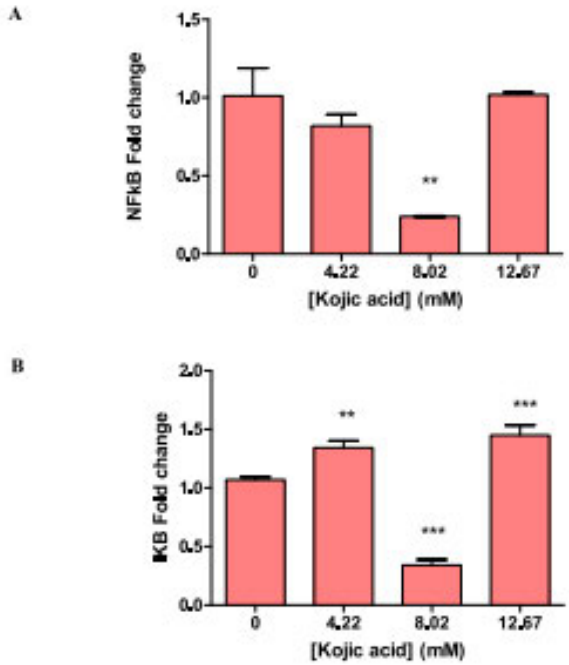

Fig. 7. Effects of KA on inflammatory markers *NFκB* (A) *and IκB* (B) gene expression in HepG2 cells. The data was represented as mean ± SD (n = 3).

decrease in protein carbonyl concentrations was observed (Fig. 4C). Protein and DNA were not negatively impacted by KA.

Mammalian cells have defence systems to maintain the intracellular environment's equilibrium (Basak et al., 2017). Increased cell stability and activity are driven by the phosphorylation of Nrf2 (p-Nrf2) (Nguyen et al., 2009). The zinc positioned between Nrf2, and KEAP-1 may be targeted by KA, allowing Nrf2 to be released. Results revealed an increase in Nrf2 and p-Nrf2 expression (Fig. 5A & B), indicating that the release of Nrf2 from KEAP-1 may be influenced by KA chelation characteristics. In stress-induced apoptosis, Nrf2 is a modulator of the anti-apoptotic protein Bcl 2. The sustained oxidative stress experienced may have contributed to the elevated p-Nrf2 expression. However, the induction of apoptotic proteins, which led to the release of cytochrome *c* into the cytoplasm and promoted apoptosome formation, may have rendered the increase in p-Nrf2 expression insufficient. This is in agreement with the findings KA displayed neuroprotective characteristics in a mouse model by increasing Nrf2 expression (Khan et al., 2021).

Antioxidants such as CAT and SOD2 contain ARE, which is activated by the transcription factor Nrf2 (Nguyen et al., 2009). Free radicals are neutralized by CAT and SOD2 reducing peroxides to H_2O and O_2 (Basak et al., 2017; Zhang et al., 2017). A decreased expression of CAT was observed following treatment with KA, but more significantly at high concentrations. The amount of MDA adducts formed is correlated with the inhibition of cellular antioxidant response and lipid peroxidation (Fig. 5C). This is consistent with other findings showing that KA deactivated the regulatory protein CAT in the rat liver and kidney (Kotyzova et al., 2004). The GPx enzyme reduces peroxides and converts glutathione to glutathione disulphide. The gene expression of *GPx* was elevated at KA concentrations with increased MDA production (low concentrations) (Fig. 5D). The study results displayed a linear relationship between oxidative stress and antioxidant responses in HepG2 cells.

In response to stimuli, cells activate the MAPK signalling pathway, which promotes inflammatory response, antioxidant response, cell survival, and cell death (Cuenda and Rousseau, 2007; Shaulian and Karin, 2002; Weston and Davis, 2002; Kammeyer and Luiten, 2015). The MAPK proteins come into focus because of KA's part in the suppression of tyrosinase activity by copper chelation. To understand the cell's response to KA toxicity, the expression of MAPK proteins JNK 1/2 and p38, which influence cellular regulation, was examined. The p38 and JNK 1/2 proteins are involved in the generation of cytokines,

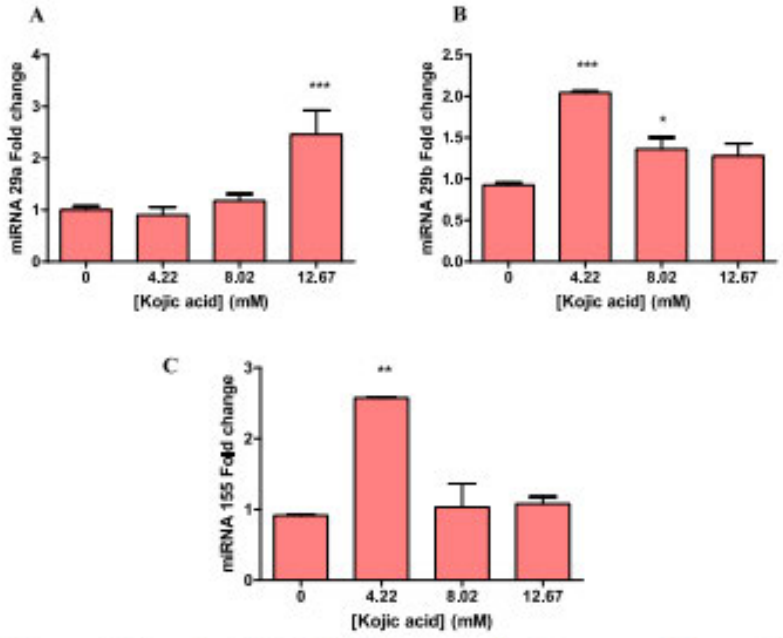

Fig. 8. KA effects on miRNA expression in HepG2 cells following treatment. The data was represented as mean ± SD (n = 3).

inflammatory responses, and apoptosis (Morrison, 2012). Oxidative stress and DNA damage both trigger the activation of JNK 1/2 (Sun et al., 2009). Additionally, JNK regulates the anti-apoptotic protein, Bcl-2 (Dhanasekaran and Reddy, 2008). This association is supported by the findings of the current investigation, as shown by reduced JNK 1/2 expression, which insufficiently inhibited Bcl-2 thereby promoting apoptosis (Fig. 6A & B). Stress activates the protein kinase p38, which is signalled via upstream MAPK signalling. In HeLa cells, KA was found to increase MAPK expression (Chen et al., 2013), which is in agreement with the results obtained for p38 in HepG2 cells (Fig. 6C). Due to the presence of ROS, prolonged activation of this pathway may result in cell death (Redza-Dutordoir and Averill-Bates, 2016; Ott et al., 2007). JNK 1/2 and p38 detect stress signals, triggering the activation of cell death and reducing cell proliferation (Ayala et al., 2014). This implies that the MAPK pathway may be involved in the response to ROS after HepG2 cells were treated with KA.

Inflammation has been linked to both the initiation and development of diseases. Key NFκB pathway indicators were examined to ascertain how KA treatment affected inflammation in HepG2 cells (Fig. 8). Gene expression of *NFκB* was decreased at the IC_{50} concentration (Fig. 7A). The gene expression of *IκB* was increased with the exception of the IC_{50}. (Fig. 7B). The results indicate a significant suppression of the NFκB inflammatory pathway by KA at 8.02 mM. *In vivo* and *in vitro* studies on KA found decreased inflammatory cytokine expression following treatment (Khan et al., 2021; Li et al., 2021; Moon et al., 2001).

MiRNAs are a class of post-transcriptional repressors of gene expression (Reinhart et al., 2000). KA significantly increased *miRNA-29a* expression at higher concentrations. At higher concentrations, apoptosis and inflammation did not occur (Fig. 8A). The result is consistent with the literature, which indicates that *miRNA-29a* decreases NFκB production in *miRNA-29a* transgenic mice and has anti-apoptotic effects on cells (Tiao et al., 2014).

In C57BL/6 mice, dysregulation of *miRNA-29b* contributes to the development of fibrosis (Su et al., 2019). Additionally, *miRNA-29b* regulates oxidative stress, reduces cell viability, and promotes apoptosis (Hou et al., 2017; Engedal et al., 2018). *MiRNA-29b* has pro-apoptotic properties which were reflected by an increase in expression at KA concentrations shown to undergo apoptosis (Fig. 8B).

By examining the expression of *miRNA-155* in treated HepG2 cells, the miRNA regulation of oxidative stress and apoptosis was evaluated. *MiRNA-155* promotes ROS generation by suppressing antioxidant responses by SOD2 and CAT. NFκB and MAPK signalling was found to regulate *miRNA-155* expression (Wang et al., 2015). However, NFκB is also a target of *miRNA*-155 by controlling IKKβ and IKKε expression (Ma et al., 2011). This results in the repression of NFκB activation which is consistent with the increased *miRNA-155* expression and decreased NFκB expression. *MiRNA-155* also stimulated cell proliferation in pancreatic cancer cells (T-rex and K-ras cells) by the inhibition of FOXO3a (Wang et al., 2015). Hence KA upregulated *miRNA-155* expression at 4.22 mM; however, at higher concentrations, no significant changes were observed (Fig. 8C). Notably, the greatest increase in MDA levels and *miRNA-155* occurred at a concentration of 4.22 mM, linking the effects of oxidative stress with miRNA expression.

5. Conclusion

The data indicate that KA has cytotoxic potential in HepG2 cells. According to studies, KA functions as an antioxidant at very low concentrations (Saeedi et al., 2019). Compared to the KA concentrations in cosmetics, the concentrations of KA found to cause oxidative stress were much higher. Through the induction of oxidative stress, HepG2 cells were vulnerable to KA toxicity. The study indicated that at the treated concentrations, there was no detectable DNA or protein damage. The opposite is true—KA has demonstrated potential hepatoprotective effects. In the presence of oxidative stress, MAPK proteins initiate inflammation. KA reduced inflammatory markers while increasing the stress-activated protein kinase p38.

The evaluation of cosmetic products was found to contain KA concentrations between 1 and 2%. Due to health concerns raised by published research on the relationship between thyroid and skin sensitization, the Scientific Committee on Scientific Products (SCCP) of the European Commission established a maximum allowable concentration of 1% in skincare products (SCCP, 2021).

It was discovered that the concentration of KA used as a food preservative in seafood was between 0.25% and 0.6%. In terms of ingestion, KA is a fermentation by-product in food and is found in small quantities. According to earlier studies, the LD_{50} dose for mammals is 1 g/kg (Brtko et al., 2004). The distribution and absorption of KA from dermal penetration were determined to be insignificant. Therefore, the distributed concentration of KA will be greatly reduced by KA metabolism.

The study findings showed that, despite elevated levels of oxidative stress and apoptosis, the impact on liver health in terms of DNA damage, protein oxidation, and inflammation was negligible. Notably, the KA concentrations needed to produce these effects were significantly higher than those typically utilized, necessitating more research based on dermal and oral exposures.

Credit author statement

KS: Conceptualization, Methodology, Investigation, Writing- Original draft preparation; **TG**: Investigation, Writing- Reviewing and Editing; **NSA**: Methodology, Writing- Reviewing and Editing; **AC**: Conceptualization, Supervision, Funding, Writing- Reviewing and Editing.

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Ethical statement

The study was conducted on a commercially available cell line. This was an *in vitro* laboratory study. Hence, no ethical considerations are involved.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2023.107221.

References

Abdul, N.S., Nagiah, S., Chuturgoon, A.A., 2016. Fusaric acid induces mitochondrial stress in human hepatocellular. Toxicon 119, 336–344.
Ayala, A., Munoz, M.F., Arguelles, S., 2014. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. 2014 Oxid. Med. Cell. Longev. 1–31.
Azami, F., Tazikeh-Lemeski, E., Mahmood-Janlou, M.-A., 2017. Kojic acid effect on the inhibitory potency of tyrosinase. J. Chem. Health Risks 7, 147–155.
Basak, P., Sadhukhan, P., Sarkar, P., Sil, P.C., 2017. Perspectives of the Nrf-2 signaling pathway in cancer progression and therapy. Toxicol Rep 4, 306–318.
Belsito, D.V., Hill, R.A., Klaassen, C.D., Liebler, D.C., 2009. TENTATIVE SAFETY ASSESSMENT Kojic Acid as Used in Cosmetics, pp. 1–68.

Brtko, J., Rondahl, L., Fickova, M., Hudecova, D., Eybl, V., Uher, M., 2004. Kojic acid and its derivatives: history and present state of art. Cent. Eur. J. Publ. Health 12 (Suppl. I), S16–S18.
Chaudhari, U., Nemade, H., Sureshkumar, P., Vinken, M., Ates, G., Rogiers, V., Hescheler, J., Hengstler, J.G., Sachinidis, A., 2018. Functional cardiotoxicity assessment of cosmetic compounds using human-induced pluripotent stem cell-derived cardiomyocytes. Arch. Toxicol. 92, 371–381.
Chen, Y.-H., Lu, P.-J., Hulme, C., Shaw, A.Y., 2013. Synthesis of kojic acid-derived copper-chelating apoptosis inducing agents. Med. Chem. Res. 22, 995–1003.
Choi, H., Kim, K., Han, J., Choi, H., Jin, S.H., Lee, E.K., Shin, D.W., Lee, T.R., Lee, A.-Y., Noh, M., 2012. Kojic acid-induced IL-6 production in human keratinocytes plays a role in its anti-melanogenic activity in skin. J. Dermatol. Sci. 66, 207–215.
Cooke, M.S., Evans, M.D., Dizdaroglu, M., Lunec, J., 2003. Oxidative DNA damage: mechanisms, mutation, and disease. Faseb. J. 17, 1195–1214.
Cuenda, A., Rousseau, S., 2007. p38 MAP-Kinases pathway regulation, function and role in human diseases. Biochim. Biophys. Acta Mol. Cell Res. 1773, 1358–1375.
Dalle-donne, I., Rossi, R., Giustarini, D., Milzani, A., Colombo, R., 2003. Protein carbonyl groups as biomarkers of oxidative stress. Clin. Chim. Acta 329, 23–38.
Dexheimer, P.J., Cochella, L., 2020. MicroRNAs: from mechanism to organism. Front. Cell Dev. Biol. 8.
Dhanasekaran, D.N., Reddy, E.P., 2008. JNK signaling in apoptosis. Oncogene 27, 6245–6251.
Elmore, S., 2007. Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35, 495–516.
Engedal, N., Žerovnik, E., Rudov, A., Galli, F., Olivieri, F., Procopio, A.D., Rippo, M.R., Monsurrò, V., Betti, M., Albertini, M.C., 2018. From oxidative stress damage to pathways, networks, and autophagy via MicroRNAs. Oxid. Med. Cell. Longev., 4968321, 2018.
Feoktistova, M., Geserick, P., Leverkus, M., 2016. Crystal violet assay for determining viability of cultured cells, 2016 Cold Spring Harb. Protoc. 343–346.
Hamajima, H., Matsunaga, H., Fujikawa, A., Sato, T., Mitsutake, S., Yanagita, T., Nagao, K., Nakayama, J., Kitagaki, H., 2016. Japanese traditional dietary fungus koji Aspergillus oryzae functions as a prebiotic for Blautia coccoides through glycosylceramide: Japanese dietary fungus koji is a new prebiotic. SpringerPlus 5, 1–10.
Higa, Y., Kawabe, M., Nabae, K., Toda, Y., Kitamoto, S., Hara, T., Tanaka, N., Kariya, K., Takahashi, M., 2007. Kojic acid -absence of tumor-initiating activity in rat liver, and of carcinogenic and photo-genotoxic potential in mouse skin. J. Toxicol. Sci. 32, 143–159.
Hou, M., Zuo, X., Li, C., Zhang, Y., Teng, Y., 2017. Mir-29b regulates oxidative stress by targeting SIRT1 in ovarian cancer cells. Cell. Physiol. Biochem. 43, 1767–1776.
Kammeyer, A., Luiten, R.M., 2015. Oxidation events and skin aging. Ageing Res. Rev. 21, 16–29.
Khan, A., Park, T.J., Ikram, M., Ahmad, S., Ahmad, R., Jo, M.G., Kim, M.O., 2021. Antioxidative and anti-inflammatory effects of kojic acid in aβ-induced mouse model of alzheimer's disease. Mol. Neurobiol. 58, 5127–5140.
Kim, D., Park, G.B., Hur dae, Y., 2014. Apoptotic signaling through reactive oxygen species in cancer cells. World J. Immunol. 4, 158–173.
Kim, D.H., Hwang, J.S., Baek, H.S., Kim, K.J., Lee, B.G., Chang, I., Kang, H.H., Lee, O.S., 2003. Development of 5-[(3-aminopropyl)phosphinooxy]-2-(hydroxymethyl)-4H-pyran-4-one as a novel whitening agent. Chem. Pharm. Bull. 51, 113–116.
Kim, J.H., Campbell, B.C., Chan, K.L., Mahoney, N., Haff, R.P., 2013. Synergism of antifungal activity between mitochondrial respiration inhibitors and kojic acid. Molecules 18, 1564–1581.
Kotyzova, D., Eybl, V., Koutensky, J., Brtko, J., Glattre, E., 2004. Effects of kojic acid on oxidative damage and on iron and trace element level in iron-overloaded mice and rats. Cent. Eur. J. Publ. Health 12 (Suppl. I), S41–S44.
Kundukad, B., Udayakumar, G., Grela, E., Kaur, D., Rice, S.A., Kjelleberg, S., Doyle, P.S., 2020. Weak acids as an alternative anti-microbial therapy. Biofilms 2, 100019.
Lajis, A.F.B., Hamid, M., Ariff, A.B., 2012. Depigmenting effect of Kojic acid esters in hyperpigmented B16F1 melanoma cells, 2012 J. Biomed. Biotechnol. 1–9.
Li, T.-X., Liang, J.-X., Liu, L.-L., Shi, F.-C., Jia, X.-W., Li, M.-H., Xu, C.-P., 2021. Novel kojic acid derivatives with anti-inflammatory effects from Aspergillus versicolor. Fitoterapia 154, 105027.
Liguori, I., Russo, G., Curcio, F., Bulli, G., Aran, L., Della-Morte, D., Gargiulo, G., Testa, G., Cacciatore, F., Bonaduce, D., Abete, P., 2018. Oxidative stress, aging, and diseases. Clin. Interv. Aging 13, 757–772.
Ma, X., Becker Buscaglia, L.E., Barker, J.R., Li, Y., 2011. MicroRNAs in NF-kappaB signaling. J. Mol. Cell Biol. 3, 159–166.
Mattson, M.P., 2008. Hormesis defined. Ageing Res. Rev. 7, 1–7.
Moon, K.Y., Ahn, K.S., Lee, J., Kim, Y.S., 2001. Kojic acid, a potential inhibitor of NF-kappaB activation in transfectant human HaCaT and SCC-13 cells. Arch Pharm. Res. 24, 307–311.
Morrison, D.K., 2012. MAP kinase pathways. Cold Spring Harbor Perspect. Biol. 4, a011254.
Nguyen, T., Nioi, P., Pickett, C.B., 2009. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. J. Biol. Chem. 284, 13291–13295.
Niwa, Y., Akamatsu, H., 1991. Kojic acid scavenges free radicals while potentiating leukocyte functions including free radical generation. Inflammation 15, 303–315.
Nurunnabi, T.R., Al-Majmaie, S., Nakouti, I., Nahar, L., Rahman, S.M., Sohrab, M.H., Billah, M.M., Ismail, F.M., Sharples, G.P., Sarker, S.D., 2018. Antimicrobial activity of kojic acid from endophytic fungus Colletotrichum gloeosporioides isolated from Sonneratia apetala, a mangrove plant of the Sundarbans. Asian Pac. J. Tropical Med. 11, 350.
Oncul, S., Karakaya, G., Dilsiz Aytemir, M., Ercan, A., 2019. A kojic acid derivative promotes intrinsic apoptotic pathway of hepatocellular carcinoma cells without incurring drug resistance. Chem. Biol. Drug Des. 1–10.
Ott, M., Gogvadze, V., Orrenius, S., Zhivotovsky, B., 2007. Mitochondria, oxidative stress and cell death. Apoptosis 12, 913–922.
Owolabi, J.O., Fabiyi, O.S., Adelakin, L.A., Ekwerike, M.C., 2020. Effects of skin lightning cream agents-hydroquinone and kojic acid, on the skin of adult female experimental rats. Clin. Cosmet. Invest. Dermatol. 283–289.
Redza-Dutordoir, M., Averill-Bates, D.A., 2016. Activation of apoptosis signalling pathways by reactive oxygen species. Biochim. Biophys. Acta Mol. Cell Res. 1863, 2977–2992.
Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., Horvitz, H.R., Ruvkun, G., 2000. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 403, 901–906.
Rodrigues, A.P., Carvalho, A.S., Santos, A.S., Alves, C.N., Do Nascimento, J.L., Silva, E. O., 2011. Kojic acid, a secondary metabolite from Aspergillus sp., acts as an inducer of macrophage activation. Cell Biol. Int. 35, 335–343.
Saeedi, M., Eslamifar, M., Khezri, K., 2019. Kojic acid applications in cosmetic and pharmaceutical preparations. Biomed. Pharmacother. 110, 582–593.
Sánchez-valle, V., C Chavez-tapia, N., Uribe, M., Méndez-Sánchez, N., 2012. Role of oxidative stress and molecular changes in liver fibrosis: a review. Curr. Med. Chem. 19, 4850–4860.
SCCP, 2021. Opinion on Kojic Acid. European Commission, pp. 1–85.
Shaulian, E., Karin, M., 2002. AP-1 as a regulator of cell life and death. Nat. Cell Biol. 4, E131–E136.
Singh, A., Bhat, T., Sharma, O., 2011. Clinical biochemistry of hepatotoxicity. J. Clin. Toxicol. S 4, 2161-0495.
Stratford, M., Anslow, P., 1998. Evidence that sorbic acid does not inhibit yeast as a classic 'weak acid preservative. Lett. Appl. Microbiol. 27, 203–206.
Su, W.-H., Wang, C.-J., Hung, Y.-Y., Lu, C.-W., Ou, C.-Y., Tseng, S.-H., Tsai, C.-C., Kao, Y.-T., Chuang, P.-C., 2019. MicroRNA-29a exhibited pro-angiogenic and anti-fibrotic features to intensify human umbilical cord mesenchymal stem cells-renovated perfusion recovery and preventing against fibrosis from skeletal muscle ischemic injury. Int. J. Mol. Sci. 20, 1–24.
Sun, Z., Huang, Z., Zhang, D.D., 2009. Phosphorylation of Nrf2 at multiple sites by MAP kinases has a limited contribution in modulating the Nrf2 dependent antioxidant response. PLoS One 4, 1–9.
Tamura, T., Mitsumori, K., Totsuka, Y., Wakabayashi, K., Kido, R., Kasai, H., Nasu, M., Hirose, M., 2006. Absence of *in vivo* genotoxic potential and tumor initiation activity of Kojic acid in the rat thyroid. Toxicology 222, 213–224.
Tiao, M.-M., Wang, F.-S., Huang, L.-T., Chuang, J.-H., Kuo, H.-C., Yang, Y.-L., Huang, Y.-H., 2014. MicroRNA-29a protects against acute liver injury in a mouse model of obstructive jaundice via inhibition of the extrinsic apoptosis pathway. Apoptosis 19, 30–41.
Wang, P., Zhu, C.-F., Ma, M.-Z., Chen, G., Song, M., Zeng, Z.-L., Lu, W.-H., Yang, J., Wen, S., Chiao, P.J., 2015. Micro-RNA-155 is induced by K-Ras oncogenic signal and promotes ROS stress in pancreatic cancer. Oncotarget 6, 21148–21158.
Weston, C.R., Davis, R.J., 2002. The JNK signal transduction pathway. Curr. Opin. Genet. Dev. 12, 14–21.
Wiesmüller, L., Ford, J.M., Schiestl, R.H., 2002. DNA damage, repair, and diseases. J. Biomed. Biotechnol. 2, 45–45.
Yang, H., Zhang, W., Pan, H., Feldser, H.G., Lainez, E., Miller, C., Leung, S., Zhong, Z., Zhao, H., Sweitzer, S., Considine, T., Riera, T., Suri, V., White, B., Ellis, J.L., Vlasuk, G.P., Loh, C., 2012. SIRT1 activators suppress inflammatory responses through promotion of p65 deacetylation and inhibition of NF-κB activity. PLoS One 7, e46364.
Yin, B., Whyatt, R.M., Perera, F.P., Randall, M.C., Cooper, T.B., Santella, R.M., 1995. Determination of 8-hydroxydeoxyguanosine by an immunoaffinity chromatography-monoclonal antibody-based ELISA. Free Radic. Biol. Med. 18, 1023–1032.
Zhang, J., Chen, H., Chen, M., Wang, H., Wang, Q., Song, X., Hao, H., Feng, Z., 2017. Kojic acid-mediated damage responses induce mycelial regeneration in the basidiomycete Hypsizygus marmoreus. PLoS One 12, 1–20.
Zilles, J.C., Dos Santos, F.L., Kulkamp-Guerreiro, I.C., Contri, R.V., 2022. Biological activities and safety data of kojic acid and its derivatives: a review. Exp. Dermatol. 31, 1500–1521.

Addendum B: Guidelines for thesis submission

In the CHS Handbook the rules for a PhD thesis are not in one place; they are stated in DR8 a i & ii, DR9 c and CHS 16. DR8 a i & ii and direct that a thesis be presented in the standard format together with one published paper or an unpublished manuscript that has been submitted to an accredited journal, arising from the doctoral research. CHS16 (thesis by publications states that the thesis may comprise of at least three published papers or in press in accredited journals; such papers must have the student as the prime author. The same CHS16 provides for a thesis by manuscripts that may have at least 3 papers with the student as the prime author that have not yet been published but are in the form of manuscripts; at least two of such papers must constitute original research. In both cases (thesis by publications and manuscripts), there must be introductory and concluding integrative material sections.

The standard type thesis is being phased out in many African countries in favour of the other options that originate from the Scandinavian countries. While this format ensures that all details of the work done for the doctoral degree are captured and thoroughly interrogated, they often remain as grey literature which is mainly useful to other students, usually within the same university, although with digitization of theses, such work may become more accessible beyond the source university. Apart from the risk of losing good work because of it not being on the public domain, as students rarely publish such work after graduating, this approach denies the college additional productivity units (PUs) emanating from publications.

The thesis by publication encourages students to publish key aspects of their doctoral research as they will not graduate if the papers are not published or in press. This approach ensures that the work of the student enters the public domain before the thesis is examined, providing the examiner with some assurance of prior peer review. The thesis must constitute a full study of the magnitude expected of a PhD with the papers providing a sound thread or storyline. Furthermore, the college maximizes the students' work as PUs are awarded for the papers as well as for graduating. However, this approach may negatively affect throughput and frustrate students as they cannot graduate unless all the papers are published or in press, in addition to the synthesis chapter demonstrating the story line of the thesis.

The option of a thesis by manuscripts ensures that students make efforts to start publishing. The risk of not passing because of failure to publish all papers (as in the thesis by publication) does not exist under this option. However, the PUs emanating from publications from the doctoral work are not guaranteed as the submitted papers may eventually be rejected. Thus there is a possibility of the doctoral work remaining on the university library shelves as is the case for the standard thesis format. The standard thesis does have the advantage that more details of the doctoral work are usually included.

In view of the above, the best option for the college is that of a thesis by publication. However, in the interim, the attractive option is that of thesis by manuscripts, as it provides the possibility of publication without putting the student at risk of delayed graduation when some of the manuscripts are not published/accepted, which also disadvantages the college in terms of PU earnings. The standard thesis option should ultimately be phased out for the stated reasons and students are not encouraged to present their theses in that format. Consequently this document does not describe the standard thesis.

Addendum C: Ethical approval letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

05 July 2023

Miss Kimera Tamzin Suthiram (216042836)
School of Laboratory Medicine & Medical Science
Howard College

Dear Miss Suthiram,

Protocol reference number: BREC/00005725/2023
Project title: An investigation into the mitochondrial toxicity of Kojic acid associated with antioxidant responses, and inflammation in melanoma cells (SK-MEL-1)
Degree: PhD

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 05 July 2023. Please ensure that any outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is valid for one year from 05 July 2023. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on RIG on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2020) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 11 July 2023.

Yours sincerely,

[redacted]

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

Addendum D: Supplementary information

The following graph displays MTT results for concentrations used to treat SK-MEL-1 cells.

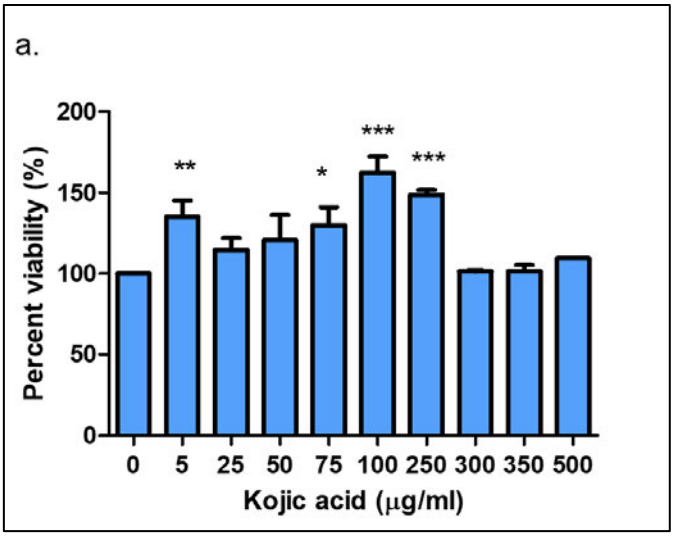

Figure S1: Cell viability was increased at all concentrations over a 24 h period. Results are represented as mean fold-change ± SD (n = 3), where ***p < 0.001, **p < 0.01 and *p < 0.05.