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Pseudomonas aeruginosa's redox-active virulence factor pyocyanin and its genotoxic and cytotoxic impact on eukaryotic cells

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Submitted in accordance with the requirements for the degree of

Masters of Science by Research

York St John University

School of Science, Technology and Health

March 2025

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Acknowledgements

First and foremost, I would like to express my deepest gratitude to my supervisor, Dr Adi Baumgartner, for his advice, time, and seemingly endless patience throughout the duration of my MRes. Thank you for always making time for me in your busy schedule, pushing me to think critically, and, most importantly, for the many laughs and conversations over the years which have kept me sane.

To Andy, Thelma, Amy, and Ash, the YSJ Biomedical Science lab technicians, thank you for all of the hard work you do to keep things running smoothly in the labs. Thank you for your endless knowledge and expertise, for always being willing to help me whenever I needed it, and for the countless laughs that made long days in the lab far more enjoyable. I couldn't have done this without you.

Lastly, a very special thanks to my partner, Sean. We met only a short while before I began my MRes but your support was unwavering from the start. You supported me emotionally, pushed me when things got tough, and, most importantly, you were always happy to listen to me ramble about my research, even if you didn't always understand it. Your patience, encouragement, and belief in me have helped me immensely and I will always be endlessly grateful for everything you've done.

Abstract

Pseudomonas aeruginosa is an opportunistic pathogen, widely known for its chronic and severe infections in immunocompromised individuals, such as those with cystic fibrosis (CF). Pyocyanin, a virulence factor of *P. aeruginosa*, plays an important role in these infections. A redox-active phenazine pigment, pyocyanin promotes virulence via the formation of reactive oxygen species (ROS). ROS formation causes oxidative stress in cells, leading to DNA damage, compromised genomic stability, and increased risk of mutation accumulation and, subsequently, cancer. While pyocyanin's redox abilities have been widely studied, its genotoxic effects on host cells remain unexplored. This study aims to explore the potential genotoxic and cytotoxic effects of pyocyanin on eukaryotic cells, focusing on human lymphocytes as a surrogate. Cell viability was assessed via the CCK-8 assay to determine the cytotoxic potential of pyocyanin. Single- and double-strand breaks were assessed via the Comet assay and PicoGreen assay to measure pyocyanin's genotoxic effects. Pyocyanin was found to have a biphasic dose-response relationship with cell viability, with increased viability at low concentrations and cytotoxicity at high concentrations. Cell viability was enhanced over time up to concentrations of 10 µM pyocyanin. A dose-response increase in DNA damage was seen, with significant damage being reported across all concentrations. Evidence was also found to suggest that pyocyanin is an intercalating agent, producing falsely increased percentages of double-stranded DNA results in the PicoGreen assay. Overall, the findings highlighted a hormetic effect of pyocyanin which has not been previously established and its ability to induce DNA damage at low concentrations raises concerns about its impact on host cells, especially in those with chronic *P. aeruginosa* infections. Further research is necessary to investigate the genotoxic effects of long-term exposure to pyocyanin in addition to its hormetic effects, which may have therapeutic potential.

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Abbreviations

8-oxoG	8-Oxoguanine
AP-1	Activator Protein 1
ATP	Adenosine Triphosphate
Bcl-2	B-Cell Lymphoma 2
BER	Base Excision Repair
cAMP.	Cyclic Adenosine Monophosphate
CCK-8	Cell Counting Kit-8
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
COPD	Chronic Obstructive Pulmonary Disease
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DSB	Double-Strand Break
dsDNA	Double-Stranded DNA
EtBr	Ethidium Bromide
ETC	Electron Transport Chain
FBS	Foetal Bovine Serum
FRET	Fluorescence Resonance Energy Transfer
GSH	Glutathione
H2O2	Hydrogen Peroxide
IL-8	Interleukin-8
LMP	Low Melting Point
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NC	Negative Control
NF-κB	Nuclear Factor Kappa B
NMP	Normal Melting Point
Nrf2	Nuclear Factor Erythroid 2-Related Factor 2
OTM	Olive Tail Moment
P. aeruginosa	*Pseudomonas aeruginosa*
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PC	Positive Control
PYO	Pyocyanin
QS	Quorum Sensing
ROS	Reactive Oxygen Species
SSB	Single-Strand Break
SSF	Strand Scission Factor
TD25	Toxic Dose 25
TDNA%	Tail DNA Percentage
TNF-α	Tumour Necrosis Factor Alpha
VAP	Ventilator Associated Pneumonia
V-ATPase	Vacuolar ATPase

1.0 Introduction

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa, a Gram-negative, facultative anaerobic bacterium, is an opportunistic pathogen, which poses no threat to healthy individuals but becomes life-threatening in those who are immunocompromised. *P. aeruginosa* is commonly associated with nosocomial infections, causing acute or chronic infections in individuals with cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), bronchiectasis, burns, those undergoing chemotherapy, and those with ventilator-associated pneumonia (VAP) including VAP caused by COVID-19 (Garcia-Nuñez et al. 2017; Lansbury et al. 2020; Oliver 2000; Paprocka et al. 2022; Vidaillac and Chotirmall 2021). Treatment of *P. aeruginosa* is extremely difficult, due to its array of antibiotic resistance determinants and rapid mutation inducing strong antibiotic resistance, with infections being frequently associated with poor clinical outcomes (Blomquist and Nix 2020).

The success of *P. aeruginosa*'s pathogenicity can be attributed to a diverse arsenal of virulence factors that play a significant role in colonisation, survival, invasion, and defence. Among these virulence factors, the production of secondary metabolites plays a key role. One such secondary metabolite which is essential for the bacteria's pathogenicity is the phenazine pigment pyocyanin (PYO) (Schroeder, Brooks, and Brooks 2017; Muller, Li and Maitz 2009).

1.2 Pyocyanin: A Multifaceted Virulence Factor

The redox-active phenazine pigment PYO is produced by *P. aeruginosa* as a secondary metabolite via a complex process involving multiple enzymes, regulated by various environmental factors such as iron availability, oxygen levels, and quorum sensing (Mavrodi et al. 2001). PYO has been discovered in approximately 90-95% of *P. aeruginosa* strains and strains deficient in this virulence factor show a reduced ability to induce cell death in neutrophils when compared to PYO-proficient strains (Usher *et al.* 2002; Lau *et al*. 2004; Ran, Hassett and Lau 2003). Pyocyanin's role in *P. aeruginosa* is an essential one, contributing to the survival, propagation, and pathogenicity of the bacteria via multiple roles.

One role of PYO is to act as an alternative electron acceptor for energy generation. Under anaerobic conditions, such as biofilms or within the mucus-filled lungs of CF patients, PYO generates ATP via oxidative phosphorylation, enabling respiration to continue (Managò et al. 2015). In biofilms, this creates a pyocyanin electrocline, a redox potential gradient which can extend hundreds of micrometres beyond the biofilm's surface, resulting in a layer of PYO which is maintained in its reduced state proximal to the biofilm (Fernanda Jiménez Otero, Newman and Tender 2023). Soluble iron (Fe^{2+}) is essential for biofilm formation and has been found to be positively correlated with the formation of the PYO electrocline. Iron is often limiting in the human body, and it has been suggested that an additional role of PYO is scavenging and reducing Fe^{3+} near the biofilm surface (Koley et al. 2011). In CF patients, CF epithelial cells will more readily release iron, suggesting this mechanism of PYO may have greater consequences in CF patients (Reid, Anderson and Lamont 2008).

Moreover, PYO-induced apoptosis of host immune cells leads to the release of extracellular matrix components, providing a structural scaffold for the biofilm. Additionally, PYO promotes extracellular DNA (eDNA) release from *P. aeruginosa*, which further strengthens the biofilm and promotes bacterial and immune cell adhesion (Das and Manefield 2012; Abdelaziz et al. 2023; Okshevsky and Meyer

2013). PYO is also able to interact with the quorum sensing (QS) system of *P. aeruginosa,* a communication mechanism that allows bacteria to coordinate gene expression (Li, Z. and Nair 2012). PYO can modulate the production of QS signalling molecules and affect the expression of QS-regulated genes, including those involved in virulence factor production, allowing *P. aeruginosa* to fine-tune its behaviour and adapt to changing conditions within the host (Al-Shabib et al. 2019).

1.3 Pyocyanin's Redox Activity

Due to its low molecular weight, PYO can easily permeate host biological membranes and, owing to its high level of stability, persist in host tissues for extended periods (El-Fouly *et al*. 2015). At pH 7, PYO is zwitterionic in structure, enabling it to accept electrons from reducing agents such as nicotinamide adenine dinucleotide phosphate (NADPH) and nicotinamide adenine dinucleotide (NADH). PYO then transfers these electrons to molecular oxygen, creating reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{-}\cdot$) (Mavrodi *et al*. 2001: Rada et al. 2008).

This process, as shown in Figure 1, involves the cyclic, non-enzymatic reduction of PYO, leading to the formation of hydrophenazine which then reacts with a second PYO molecule. This process creates two PYO radicals which rapidly react with molecular oxygen, regenerating the oxidised form of PYO and reducing the oxygen molecules to $O_2^{-}\cdot$, a highly reactive oxygen species. Superoxide dismutase (SOD) then converts superoxide to hydrogen peroxide (H_2O_2) which, while less reactive than superoxide, can participate in the Fenton reaction in the presence of transition metals, such as iron (Fe^{2+}), leading to the generation of the extremely reactive hydroxyl radical (•HO) (Davis and Thornalley 1983).

Usually, ROS regulate physiological cellular functions by acting as cell signalling molecules. In low concentrations, ROS induce eustress in the cell, activating diverse signalling pathways involved in the growth, proliferation, and death of cells (Pan, J. et al. 2010). However, excessive ROS overwhelm the cell and cause loss of ROS signalling and, subsequently, the damage of cellular macromolecules such as

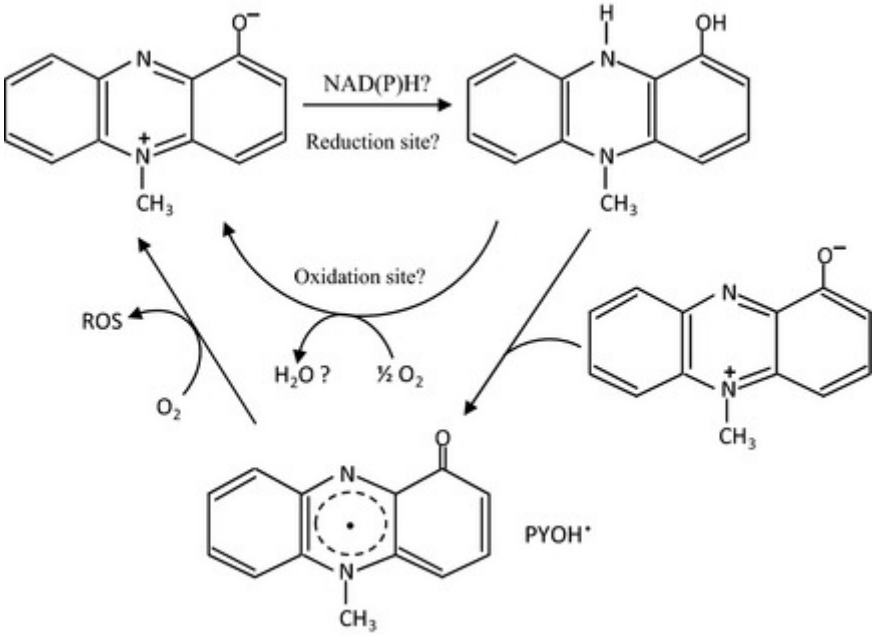

Figure 1 Pyocyanin redox cycling (Barakat et al., 2013). Oxidised PYO undergoes reduction by NAD(P)H, generating the reduced form of PYO. The reduced PYO then interacts with oxidised PYO, generating the reactive PYO radical (PYOH·). PYO undergoes auto-oxidation in the presence of oxygen (O_2), producing reactive oxygen species such as superoxide (O_2^{-}) and hydrogen peroxide (H_2O_2). Both redox states of pyocyanin are capable of interfering with the mitochondrial respiratory chain, contributing to oxidative stress within cells.

lipids, proteins, and DNA (O'Connor 2015). Continuous ROS production and disrupted cellular redox balance are the primary drivers of PYO's genotoxic and cytotoxic effects.

1.4 Pyocyanin's Genotoxic Effects on Eukaryotic Cells

Genotoxicity plays an important role in cellular dysfunction, mutation accumulation, and disease progression. While ROS are known for their ability to cause oxidative DNA damage, PYO's potential to induce genotoxic effects in eukaryotic cells remains an area lacking in research.

One of the ways ROS damage DNA is via base modification, oxidizing DNA bases directly, and guanine is particularly vulnerable. This oxidation results in the formation of 8-oxoguanine (8-oxoG), a well-known lesion that can lead to mutations (Driessens et al., 2009; Verbon, Post and Boonstra 2012). The presence of 8-oxoG can lead to errors during DNA replication, causing G:C to T:A transversions if left unrepaired (Fleming and Burrows, 2021; Shibutani et al., 1991).

In addition to base modifications, ROS may induce single-strand breaks (SSBs) in the DNA phosphate-sugar backbone. These breaks can occur directly through ROS interaction with the deoxyribose sugar or indirectly as a result of the base excision repair (BER) pathway (McDonald et al., 1993). While less severe than double-strand breaks (DSBs), SSB accumulation can overwhelm cellular repair capacity and cause genomic instability (Kuzminov 2001). DSBs can be induced by spatially proximate SSBs on opposite DNA strands or as a result of the collapse of replication forks encountering unrepaired lesions, such as persistent SSBs or bulky adducts (Driessens et al., 2009). DSBs are challenging for cells to repair accurately, and misrepair can lead to chromosomal rearrangements, deletions, and translocations, all hallmarks of genomic instability and potential contributors to carcinogenesis (Burssed et al. 2022; Qiu et al. 2017).

While these methods of genotoxicity do not have a direct link to PYO, the existing literature does show an established redox mechanism of PYO, which ultimately leads to the production of ROS (Muller 2002). It is, therefore, highly plausible that PYO-induced ROS formation could contribute to base modifications, SSBs, and DSBs, but further research is necessary to directly investigate whether the redox activity of PYO can be directly linked to the above-mentioned methods of DNA damage in eukaryotic cells.

PYO-induced oxidative stress has been shown to have a direct link to the inactivation of vacuolar (V-)ATPases, the ATP-driven proton pumps responsible for maintaining pH gradients across cellular membranes, in human lung epithelial cells (Jefferies, Cipriano and Forgac 2008; Ran, Hassett and Lau 2003). V-ATPases are crucial for the functioning of the cystic fibrosis transmembrane conductance regulator (CFTR) protein in airway epithelial cells. When inhibited, chloride ion transport is disrupted, affecting fluid balance in the airways (Quinton, 2008). A study found that 27 µg/ml (128.5 µM)PYO was enough to interfere with V-ATPase activity, and at concentrations of 50-100 µg/ml (237.8 µM - 475.6 µM) PYO, this activity was entirely diminished. This poses a significant threat to CF patients as they typically have reduced, or a lack, of CFTR. A PYO-induced diminishment of V-ATPase activity could potentially lead to a worsening of CF symptoms (Stewart-Tull and Armstrong 1972).

Host cells possess several mechanisms by which they neutralise oxidant species, such as the glutathione-peroxidase system, which neutralises hydrogen peroxide by reducing it to water and the lipid hydroperoxides to the corresponding lipid alcohol (Mills 2004). However, in the presence of PYO, glutathione (GSH) must compete for reducing agents. Additionally, some research shows that PYO can

directly oxidise glutathione, potentially enhancing PYO-induced cellular damage by providing an alternative source of reducing equivalents for PYO redox cycling, and depleting GSH levels create susceptibility to more oxidative stress and subsequent cellular damage (Muller 2011; O'Malley et al. 2004). This can be a significant issue, especially in CF patients, where GSH is already depleted.

1.5 Pyocyanin's Cytotoxic Effects on Eukaryotic Cells

While lower levels of ROS can induce genetic damage in eukaryotic cells via oxidative stress, an overproduction of these molecules can damage the cell so severely that apoptosis may occur. PYO-induced ROS have been found to activate redox-sensitive transcription factors, such as nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1). The activation of these transcription factors triggers the release of pro-inflammatory cytokines, including interleukin-8 (IL-8) and tumour necrosis factor-alpha (TNF-α) at the site of infection. In turn, immune cells are recruited to the infection site, where they are subjected to damage by PYO, reducing the efficacy of *P. aeruginosa* clearance. PYO-inflicted damage to neutrophils is one of the most significant ways this virulence factor causes damage to the host, especially in CF patients. Neutrophils have an integral role in the pathogenesis of CF, with increased numbers being found in the tissues of the airways, resulting in chronic inflammation (Lauredo et al. 1998). To kill these cells, some research suggested that PYO increases oxidative stress in the neutrophils coupled with a decrease in intracellular cyclic AMP (cAMP) concentrations. PYO concentrations as little as 10 μM were able to achieve this, with neutrophil apoptosis increasing 10-fold at concentrations of 50 μM PYO (Usher *et al*. 2002).

Another way PYO induces cytotoxicity is via interference with mitochondrial function. PYO has been shown to disrupt the electron transport chain (ETC) by accepting electrons from Complexes I and II, which results in the inhibition of Complex III. Not only does this increase oxidative stress within the cell but it also halts cellular ATP production, resulting in the depletion of cellular energy and the impairment of various cellular functions, accelerating the process of senescence and apoptosis (Managò et al. 2015).

Normally, apoptotic cells are removed by phagocytes, such as macrophages, but if apoptosis exceeds the ability of these cells, or if these cells are impaired, secondary necrosis can occur. PYO-induced impairment of these cells, as described above in the case of neutrophils, can lead to inefficient clearing of cellular contents. This can lead to secondary necrosis and the release of intracellular components, which, if not cleared, can trigger an inflammatory response and subsequent tissue death.

Ultimately, while PYO's roles in *P. aeruginosa* pathogenesis have been well documented, there is a lack of understanding of the genotoxic and cytotoxic effects of PYO on eukaryotic cells. Studying these effects is of clinical relevance, especially in the context of chronic *P. aeruginosa* infections, such as those often found in CF patients. These infections create an environment where PYO's damaging effects are likely to be amplified. The long-term consequences of chronic *P. aeruginosa* infections raise concerns about PYO's potential to cause DNA damage, accumulation of genetic mutations, and, ultimately, an increased risk of cancer development. This study aims to bridge this knowledge gap by assessing the cytotoxic and genotoxic effects of PYO. Understanding these effects can elucidate the possible long-term effects of *P. aeruginosa* in chronic infections and aid future research into the development of targeted therapies, especially those which target PYO.

1.6 Aims and Objectives

The goal of this study is to investigate the genotoxic and cytotoxic effects of pyocyanin (PYO), a virulence factor produced by *Pseudomonas aeruginosa,* on eukaryotic cells. To achieve this the study has several aims: the *in vitro* cytotoxic threshold of PYO will be determined in isolated human lymphocytes via the Cell Counting Kit-8 (CCK-8) assay to quantify cell viability following treatment with PYO; the time-dependent effects of PYO treatment on cell viability will also be assessed by the CCK-8 assay to provide insight into the dynamics of PYO-induced cytotoxicity; the research will investigate whether, and how, PYO induces DNA damage in lymphocytes. The presence of both SSBs and DSBs will be assessed with the alkaline Comet assay and the PicoGreen assay will be used to specifically measure the presence of SSBs.

2.0 Materials and Methods

2.1 Chemicals and Reagents

Pyocyanin was obtained from Selleck Chemicals, PicoGreen™ Dye Solution was obtained from Thermo Fisher Scientific, and SYBR™ Safe DNA Gel Stain was obtained from Thermo Fisher Scientific.

2.2 Collection of whole blood

Blood samples were collected from healthy volunteers into lithium heparin vacutainers by a trained phlebotomist following standardised procedures. Written informed consent was obtained from all volunteers prior to collection. Ethical approval was granted by The School of Science, Technology, and Health Research Ethics Committee of York St John University (STHEC0071).

2.3 Isolation of PBMCs

Heparinized blood was diluted with an equal volume of saline solution (0.9% NaCl). 6 ml of diluted blood was then layered on top of 3 ml Ficoll-Paque™ in a 15 ml Falcon tube. Following centrifugation (800 x *g*, 20 minutes, 21°C, no break), peripheral blood mononuclear cells (PBMCs) were collected using a plastic Pasteur pipette and transferred to a 15 ml falcon tube pre-filled with 6 ml saline. After centrifugation (500 x *g*, 15 minutes, 21°C, no break), supernatant was discarded, and the pellet resuspended in 900 µl phosphate-buffered saline for immediate use. For future use, the pellet was instead resuspended in 900 µl foetal bovine serum (FBS) and split into two cryo-vials (450 µl each), each pre-filled with 50 µl dimethyl sulfoxide (DMSO). Cryo-vials were frozen at -20°C overnight before being transferred to -80°C for long-term storage.

2.4 Cell Count and Viability

Cell count and viability were performed at the time of harvest for the CCK-8 assay. Following the isolation of PBMCs, 60 µl of the sample was loaded into a Via-1 Cassette (chemometec, 941-0012) containing acridine orange (AO) and 4',6-diamidino-2-phenylindole (DAPI) dyes. Cassettes were read using the NucleoCounter® NC-3000 (Chemometec 970-3002), providing a total cell count and percentage cell viability. These values were then used to calculate the appropriate dilution factor for the cell suspension stocks to ensure optimal cell density for the CCK-8 assay.

2.5 Treatments

All treatments were performed in a manner that minimised sample dilution, with the volume of added solutions or working stocks never exceeding 1% of the total sample volume.

2.5.1 PYO

Pyocyanin was dissolved in DMSO to prepare a 23.8 mM stock solution. This stock solution was stored at -20°C and protected from light. Working stock solutions were prepared ahead of time by diluting the stock solution in DMSO and were stored at -20°C. Negative control (NC) cells received an equal volume of Gibco Roswell Park Memorial Institute (RPMI) medium, and the vehicle control received an equivalent volume of DMSO.

2.5.2 H_2O_2

H_2O_2 served as positive controls (PCs). Working stock solutions were prepared ahead of time and stored at -20°C and protected from light. Stocks were prepared by diluting a 30% (w/w) H_2O_2 solution in distilled water.

2.5.3 Assay Treatments

Following a rest period of 24 hours, cells were treated with PYO at final concentrations of 0 (NC), 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, and 10 µM. H_2O_2 (PC) final concentrations used in this assay were 1, 50 and 100 µM. The PicoGreen assay was conducted with PYO concentrations of 0 (NC), 0.005, 0.01, 0.05, 0.1, 0.5, and 1 µM. H_2O_2 (PC) final concentrations used in this assay were 1 and 50 µM. The Comet assay was conducted with PYO concentrations of 0.01, 0.05, 0.1, 0.5, and 1 µM. H_2O_2 (PC) final concentrations used in this assay were 1 µM.

2.6 Experimental Procedures

2.6.1 CCK-8 Assay

Total alive/dead count was performed on isolated lymphocytes using Via1-Cassettes for the NC-3000 (chemometec 941-0012). 100 µl of cell suspension containing approximately 200,000 cells/100 µl in RPMI 1640 medium was pipetted into each well in a flat-bottomed 96-well cell culture plate. Plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 24 hours. After incubation, 1 µl of treatment was added to the wells. Three replicates were created for each treatment point. Plates were incubated with treatment for 24 or 48 hours at 37°C in a humidified atmosphere with 5% CO_2. Following incubation, 10 µl of CCK-8 reagent was added to each well, and absorbance at 450 nm was measured using a multifunction microplate reader (Varioskan LUX Multimode Microplate Reader) after incubation for 2 hours at 37°C and 5% CO_2.

2.6.2 Comet Assay

2.6.2.1 Cell treatment and lysis

A 1 ml end volume of whole blood solution was used for the comet assay. In Eppendorf tubes, 980 µl sterile 1 x HBSS, 10 µl whole blood, and 10 µl treatment were combined. Treatment points were as follows: NC (only cells and HBSS), DMSO, 1 µM H_2O_2, 10 µM H_2O_2, and PYO concentrations of 0.01, 0.05, 0.1, 0.5, and 1 µM. Eppendorf's were then incubated at 37°C for either 24 or 48 hours. Following incubation, tubes were centrifuged at 400 x *g*, 20°C for 3 minutes. 900 µl supernatant was discarded and the pellet was resuspended in 100 µl 1% low melting point (LMP) agarose. 100 µl each of cell-agarose suspension was pipetted onto two Superfrost slides pre-coated with 1% normal melting point (NMP) agarose. Slides were left to solidify on an ice box before 100 µl of 0.5% LMP agarose was pipetted onto each slide. Cells were once again left to solidify before being placed into fresh, cold lysis solution and incubated at 4°C overnight.

2.6.2.2 Alkaline Comet Assay

After lysis, slides were placed in a horizontal electrophoresis chamber and covered with fresh, cold alkaline electrophoresis buffer. The slides were then incubated at 4°C for 30 minutes for DNA unwinding. Electrophoresis was then performed at 4°C and 0.8 V/cm for 30 minutes. Following this, slides were washed with a neutralisation buffer 3x for 5 minutes. Slides were then washed with PBS for 5 minutes before being dehydrated in ice-cold methanol for 5 minutes. After airdrying, all slides were rehydrated with 100µl SYBR™ Safe DNA Gel Stain for scoring. Images were captured using a 40x objective lens with a fluorescent microscope, and images were analysed using ImageJ and the add-on tool OpenComet.

2.6.3 Fast Micromethod DNA Single-Strand-Break (PicoGreen) Assay

The methodology was adapted from the published protocol 'Fast Micromethod DNA Single-Strand Break Assay' by Schröder et al. (2006).

Isolation of PBMCs from whole blood was conducted prior to the assay. 20 µl of separated lymphocyte suspension containing 50,000 cells was pipetted into each well in a flat-bottomed, black 96-well plate. 0.2 µl of treatment was added to each well and plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 24 hours (three replicates were created for each treatment point). After incubation, plates were centrifuged (350 x *g*, 5 minutes, 21°C, no break) and the supernatant discarded. The pellet was resuspended in 20 µl deionised water before being centrifuged again (350 x *g*, 5 minutes, 21°C, no break). The supernatant was discarded, and the pellet resuspended in 20.2 µl 1x PBS buffer. 20.2 µl of PicoGreen dye solution (1:10 dilution of PicoGreen and lysis solution) was pipetted into each well and left to incubate on ice in a dark room for 40 minutes. After incubation, 200 µl unwinding solution was pipetted into each well and the plate was immediately read by a multifunction microplate reader (Varioskan LUX Multimode Microplate Reader). Plate reader specifications were as follows: the plate was read every 5 minutes for a total of 7 cycles (30 minutes total) in a kinetic loop, with the fluorescent excitation reading set to 490 nm, the bandwidth to 5 nm and the emission to 535 nm, at 20°C. The change in fluorescent kinetics was then recorded and exported to an Excel file.

2.7 Data Analysis and Statistics

Graphics were drawn and statistical analysis was conducted using GraphPad Prism 10 software. Data are represented as mean ± standard error of at least three independent experiments, each containing three replicates.

For the CCK-8 and Comet assays, the Kruskal-Wallis test was used to test for statistical significance between treatment groups. A post hoc Dunn's test was then used to compare treatment groups to the negative control (NC). For the CCK-8 assay, a paired samples T-test was also used to find any significant difference between 24- and 48-hour treatment groups.

For the PicoGreen assay, data in Figure 5 were analysed by a One-way ANOVA with a Dunnett's multiple comparisons test to find comparisons between treatment groups. Due to non-normality as determined by the Shapiro-Wilk test, data in Figure 6 were analysed by a Kruskal-Wallis test, followed by a Dunn's multiple comparison test.

Statistical significance was set to $p \leq 0.05$(*), $p \leq 0.01$(**), $p \leq 0.001$(***), and $p \leq 0.0001$(****) for all tests.

3.0 Results

3.1 Pyocyanin exhibits biphasic effects on lymphocyte viability. The cytotoxic potential of pyocyanin on isolated lymphocytes was determined by treating cells with increasing concentrations of PYO (0.005-10 µM). Cells were treated for either 24 or 48 hours and cell viability was assessed using the CCK-8 assay. After 24 hours (Figure 2a), cells treated with PYO exhibited a dose-dependent decrease in viability. Lower concentrations of PYO (0.005 and 0.01 µM) showed some increase in cell viability compared to the NC and DMSO, but this difference was not significant. Intermediate concentrations of PYO (0.05 – 1 µM) demonstrated increasingly cytotoxic effects on the cells, with 0.5 and 1 µM decreasing cell viability at rates comparable to those of 1 and 50 µM hydrogen peroxide (H_2O_2) respectively, a well-known cytotoxic compound. Higher concentrations of PYO (5 and 10 µM) produced a significant decrease in cell viability ($p<0.001$), with viability being greatly reduced compared to the highest concentration of H_2O_2 (100 µM).

Figure 2b shows the effects of pyocyanin on lymphocytes after 48 hours. The effects of PYO were similar to those after 24 hours, with increasing concentrations of pyocyanin reducing viability of lymphocytes. Intermediate concentrations of PYO (0.1-5 µM) caused increasingly cytotoxic effects on the cells, with 10 µM PYO significantly affecting cell viability ($p<0.001$). Cells treated with 0.005 and 0.01 µM PYO for 48 hours showed a significant increase in cell viability ($p<0.05$) when compared to the NC.

3.2 Effects of pyocyanin on lymphocyte survival at 24 and 48 hours. Treatment of lymphocytes with PYO for 24 and 48 hours demonstrated a time-dependent effect on cell viability (Figure 3). An increase in cell viability after 48 hours treatment when compared to 24 hours treatment was seen in all PYO treated groups, except 10 µM , with a significant difference seen at 0.05 and 5 µM PYO ($p<0.01$). In contrast, cells treated with 10 µM PYO showed no obvious difference in cell viability between 24 and 48 hours of treatment. DMSO and the positive control (100µM H_2O_2) produced no significant changes in lymphocyte survival between 24 and 48 hours.

3.3 Pyocyanin's cytotoxic threshold for lymphocytes. The toxic dose 25 (TD25) defines the point at which a substance is considered cytotoxic. Based on research by (Henderson et al., 1998), a substance can be considered cytotoxic at the concentration at which 75% of cells, or less, survive. The TD25, defined as the concentration of PYO at which cell survival is reduced to 75% relative to the negative control (NC), was determined for lymphocytes treated with pyocyanin for 24 and 48 hours. For cells treated for 24 hours (Figure 4a), the TD25 was calculated to be 0.153 µM and after 48 hours, the TD25 increased to 0.38 µM (Figure 4b), reflecting a shift in the cytotoxic threshold over time.

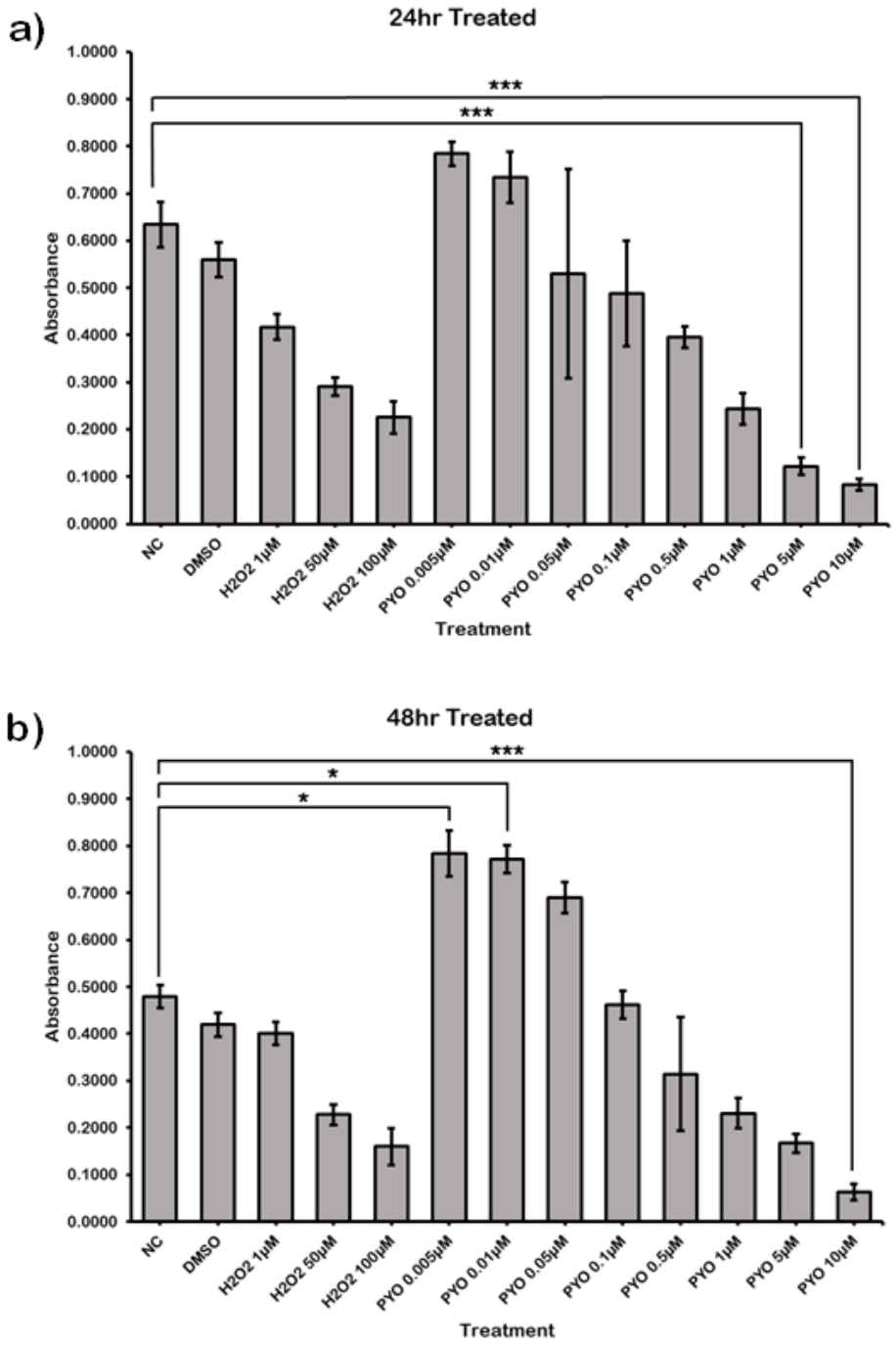

Figure 2 Cytotoxic effects of pyocyanin on isolated lymphocytes measured using the CCK-8 assay. Lymphocytes were treated with varying concentrations of pyocyanin (0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, and 10 μM), with untreated cells (NC), DMSO, and H_2O_2 (1, 50, and 100 μM) as controls for (a) 24 hours or (b) 48 hours. Absorbance values, representing cell viability, were measured at 450nm using the CCK-8 assay. Data are presented as the means ± standard error (SE). Statistical significance was determined using a Kruskal-Wallis test with post hoc Dunn's test (*p<0.05, **p<0.01, ***p<0.001, n=7).

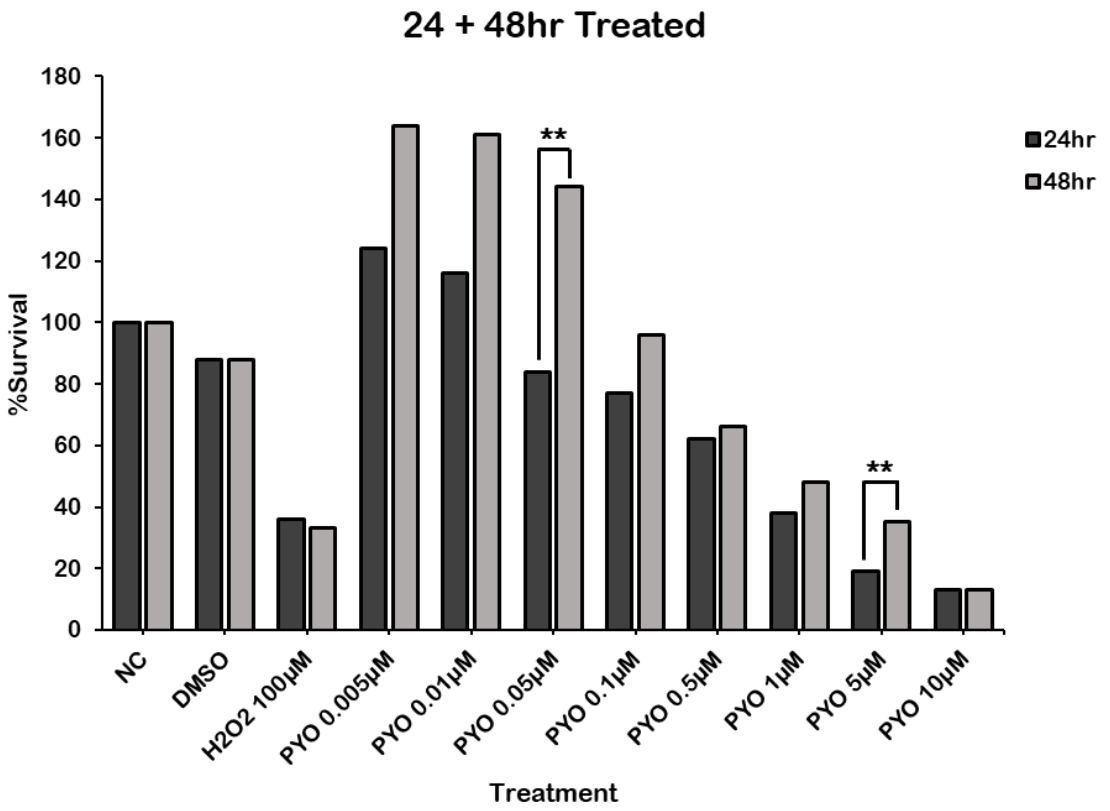

Figure 3 Effect of pyocyanin on lymphocyte viability over 24 and 48 hours, as assessed by the CCK-8 assay. Isolated lymphocytes were treated with varying concentrations of pyocyanin (0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, and 10 µM), with untreated cells (NC), DMSO, and H_2O_2 (100 µM) as controls. Data are presented as the mean survival rate of the lymphocytes relative to the negative control (expressed as a percentage). Statistical significance between 24 hour and 48 hour treated groups was determined using a paired samples T-test (**p<0.01, n=7).

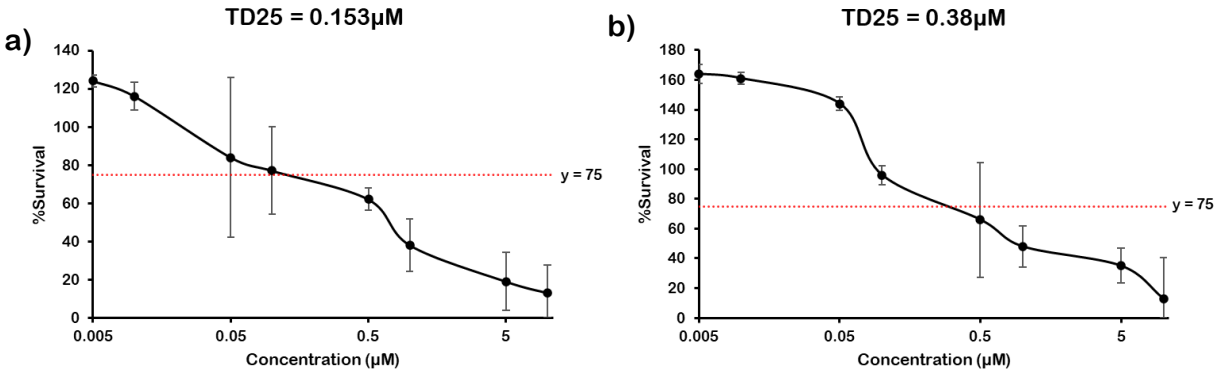

Figure 4 Determination of the toxic dose 25 (TD25) for isolated lymphocytes treated with pyocyanin. Cells were treated with varying concentrations of pyocyanin (0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, and 10 µM) for (a) 24 hours and (b) 48 hours. Viability of cells was assessed using the CCK-8 assay. Data are presented as the mean survival rate of the cells relative to the negative control (untreated cells) and expressed as a percentage. Each data point represents the mean ± standard error (SE) (n=7). Negative error bars were truncated at 0% survival where applicable to represent biological limits. Data is presented on a logarithmic scale (base 10) for clarity. The TD25, defined as the concentration at which cell survival is reduced to 75%, was calculated to be (a) 0.153µM for cells treated for 24 hours and (b) 0.38µM for cells treated for 48 hours. The intersecting line (y = 75) represents the threshold used to determine the TD25 for both treatment durations.

3.4 Effects of pyocyanin on dsDNA dynamics in lymphocytes. Figure 5 shows the time-dependent effects of pyocyanin on the percentage of dsDNA in isolated human lymphocytes. Cells were treated with increasing concentrations of PYO (0.005 – 1 µM) for 24 hours and the PicoGreen Assay was used to measure fluorescence at 5-minute intervals over the course of 30 minutes. The NC group showed a relatively stable decrease in %dsDNA over the 30 minutes, showing a reduction from 100% dsDNA to approximately 70%. There was a progressive decline in %dsDNA in the 1 µM H_2O_2 (positive control) group, reducing from 89% at t=0 to 61% at 30 minutes.

Exposure to PYO produced an immediate decrease in %dsDNA at t=0 for all PYO treated groups. The most notable was 0.01 µM PYO where, at t=0, there was a statistically significant decrease in %dsDNA when compared to the NC group ($p<0.05$). This initial decrease in %dsDNA in PYO treated groups was not dose-dependent, instead, some of the higher concentrations (0.05 and 0.1 µM PYO) produced only a minimal decrease in %dsDNA and, at 0.5 µM PYO, the initial %dsDNA surpassed that of the NC, producing a %dsDNA of 106%. Following the initial measurement at t=0, PYO treatment resulted in variable changes in %dsDNA over the 30-minute period. Some concentrations, including 0.005, 0.05, and 0.5 µM, produced a relatively stable decrease in %dsDNA, while 0.01 µM PYO showed a minor decrease up until 25 minutes. At the 30-minute interval, 0.01 µM PYO showed an increase in %dsDNA, surpassing the t=0 percentage for this concentration. Overall, at the 30-minute mark, there was no significant difference in %dsDNA for any PYO treated group when compared to the control and no dose-dependent relationship between PYO concentration and %dsDNA was apparent.

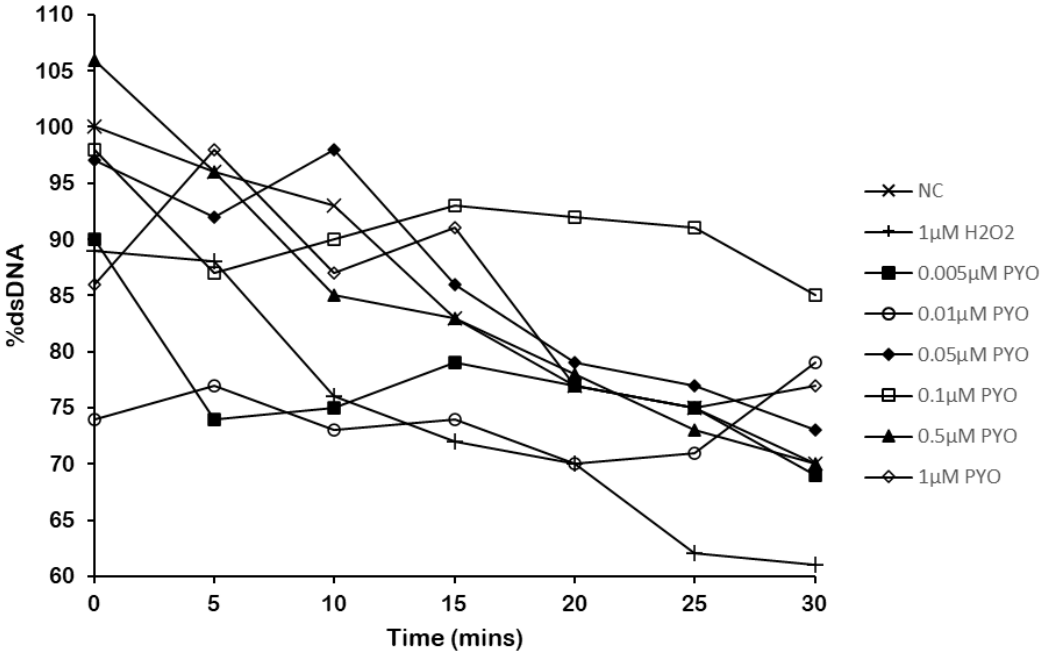

Figure 5 Time course of %dsDNA in human lymphocytes treated with pyocyanin as assessed by the Fast Micromethod DNA Single-Strand-Break Assay (PicoGreen). Isolated lymphocytes were treated for 24 hours with varying concentrations of pyocyanin (0.005, 0.01, 0.05, 0.1, 0.5, and 1 µM), with untreated cells (NC), and H_2O_2 (1 µM) as controls. Data represent the mean percentage of dsDNA in isolated lymphocytes over a 30-minute period with measurements taken every 5 minutes. Error bars are omitted for clarity. A significant decrease in %dsDNA was observed at 0.01 µM PYO at t=0 (*$p<0.05$, n=6).

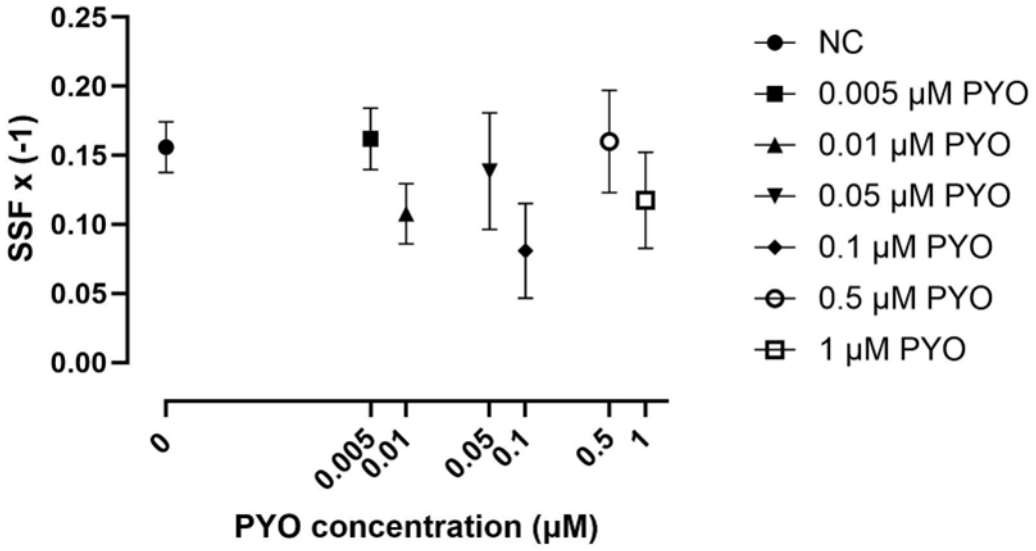

Figure 5 DNA damage in human lymphocytes after 24-hour incubation with pyocyanin. Cells were incubated with varying concentrations of pyocyanin (0.005, 0.01, 005, 0.1, 0.5, and 1 µM) with untreated cells (NC = 0 µM PYO) as a control for 24 hours. Data is expressed as the mean SSF x (-1) ± SEM (n=6). The SSF was calculated as log10(%dsDNA in the sample / %dsDNA in the control), where %dsDNA represents the percentage of double-stranded DNA remaining after treatment and unwinding (at t = 30). SSF values were multiplied by -1 for visualisation. A one-way ANOVA with Dunnett's multiple comparisons test revealed no statistically significant differences between the mean SSF x (-1) of the treatment groups and the NC (all adjusted p-values > 0.05). No trendline is shown due to the non-linear relationship between PYO concentration and SSF.

3.5 Effect of pyocyanin on DNA strand scission factor (SSF). The strand scission factor (SSF) multiplied by -1 (SSF x (-1)) was calculated to present DNA damage in isolated lymphocytes exposed to varying concentrations of PYO (0.005 – 1 µM) for 24 hours, with untreated cells (NC = 0 µM PYO) as a control. SSF x (-1) values were calculated after an unwinding period of 30 minutes. Higher SSF x (-1) values indicate more single-strand breaks and, as such, more DNA damage.

Baseline DNA damage, indicated by the mean SSF x (-1) at the NC, was approximately 0.15 (Figure 6). After the 30-minute unwinding duration, PYO treated cells showed no dose-dependent change in SSF x (-1) values. The lowest concentration of PYO (0.005 µM) showed a mean SSF x (-1) very similar, if slightly higher, to the NC group, indicating only a slight increase in overall DNA damage. The lowest SSF x (-1) value was seen in the 0.1 µM PYO treated group indicating that these cells experienced the least DNA damage. PYO The 0.005 and 0.5 µM PYO treated groups showed a slight increase in SSF x (-1) when compared to the NC (0.162 and 0.160 respectively compared to 0.156), with the 0.005, 0.05, and 0.5 µM PYO groups showing an overall increase in SSF x (-1) when compared to the 0.01, 0.1, and 1 µM PYO treated groups. Error bars indicate a large degree of variability in PYO treated groups, specifically the middle-to-high concentrations (0.05 – 1 µM). The highest concentrations of PYO, 0.5 and 1 µM, produced a similar to, or lower, SSF x (-1) than the NC, suggesting less DNA damage in these treatment groups after 30 minutes of unwinding when compared to the NC.

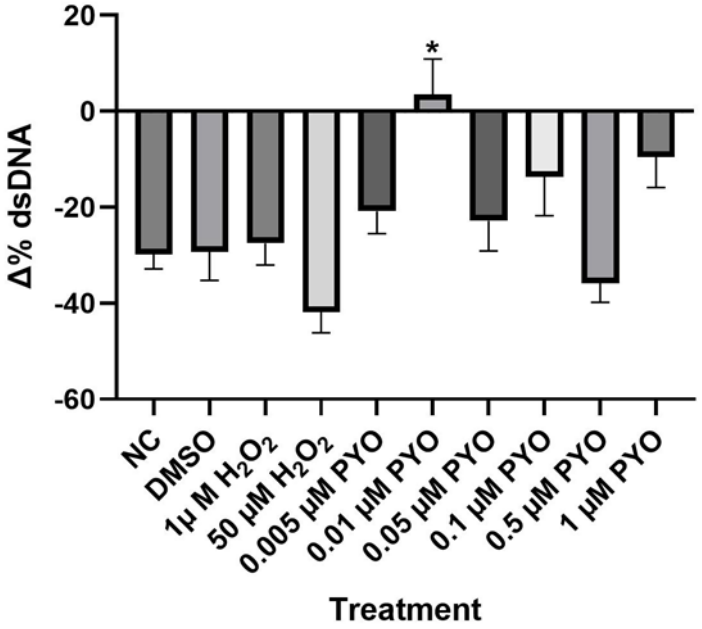

Figure 6 Change in %dsDNA in isolated lymphocytes after treatment with pyocyanin for 24 hours. Cells were incubated with varying concentrations of pyocyanin (0.005, 0.01, 005, 0.1, 0.5, and 1 µM) with untreated cells (NC) and DMSO as negative and vehicle controls, and H_2O_2 (1 and 50 µM) as positive controls. Unwinding period was 30-minutes, with readings taken every 5 minutes. Data represent the change in mean %dsDNA between 0 and 30 minutes. Error bars represent the standard error of the mean (SEM) for n=6 independent repeats. Data were analysed by Kruskal-Wallis test followed by Dunn's multiple comparison test due to violations of normality and homogeneity of variance (*p<0.05).

3.6 %dsDNA change over time. The change in the percentage of dsDNA between 0 and 30 minutes reflects the susceptibility of the DNA to unwinding after treatment with PYO. An increased change in %dsDNA over time indicates more rapid unwinding of the dsDNA, and therefore more DNA damage. Isolated lymphocytes were treated with varying concentrations of PYO (0.005 – 1 µM), H_2O_2, DMSO, or they were left untreated for 24 hours. The modified Fast Micromethod Single-Strand-Break Assay, which utilises the principle of alkaline unwinding, was employed. Figure 7 represents the change in the percentage of dsDNA in the lymphocytes over the 30-minute unwinding period. Negative Δ%dsDNA values indicate a decrease in dsDNA over the unwinding period, whereas positive values suggest an increase.

As shown in Figure 7, there was a significant increase in Δ%dsDNA for the 0.01 µM PYO treatment group when compared to the NC ($p<0.05$). This suggests that, at 0.01 µM PYO, PYO treatment led to an increase in the mean percentage of dsDNA over the unwinding period. The greatest decrease in Δ%dsDNA in the 50 µM H2O2 treated group, as expected, though this was not significant. There was no dose-dependent decrease in the change of %dsDNA in the PYO treated groups. Some PYO concentrations, 0.005, 0.05, and 0.5 µM, produced a decrease in Δ%dsDNA, suggesting an overall decrease in dsDNA and faster unwinding of the dsDNA. PYO concentrations 0.1 and 1 µM, however, produced a smaller Δ%dsDNA, with the highest concentration of PYO (1 µM) producing the smallest Δ%dsDNA of all the PYO treatments, suggesting decreased kinetics of unwinding of the dsDNA compared to all other PYO treatments.

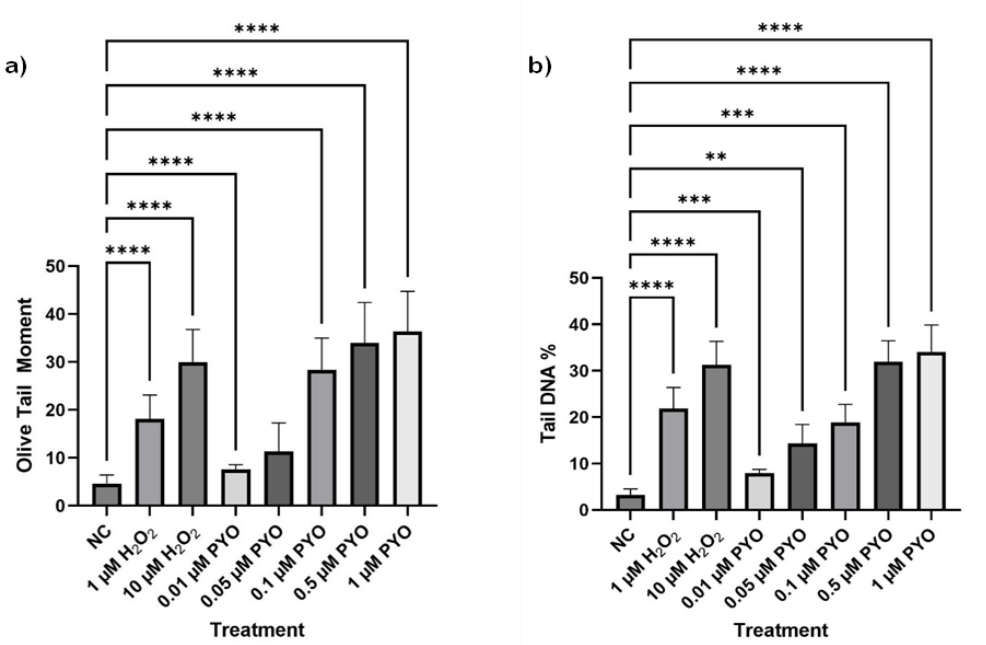

Figure 8 Pyocyanin-induced dose-dependent DNA damage in lymphocytes, as assessed by the Comet assay. Lymphocytes were treated with increasing concentrations of pyocyanin (PYO) for 24 hours before performing the alkaline Comet assay. (a) Olive tail moment and (b) Tail DNA % were measured to assess DNA damage. Hydrogen peroxide (H_2O_2) at 1 µM and 10 µM served as positive controls, while untreated cells (NC) served as the negative control. Data are presented as mean ± SEM. Statistical significance was determined using the Kruskal-Wallis test followed by Dunn's post hoc test (**p<0.01, ***p<0.001, ****p<0.0001, n=3).

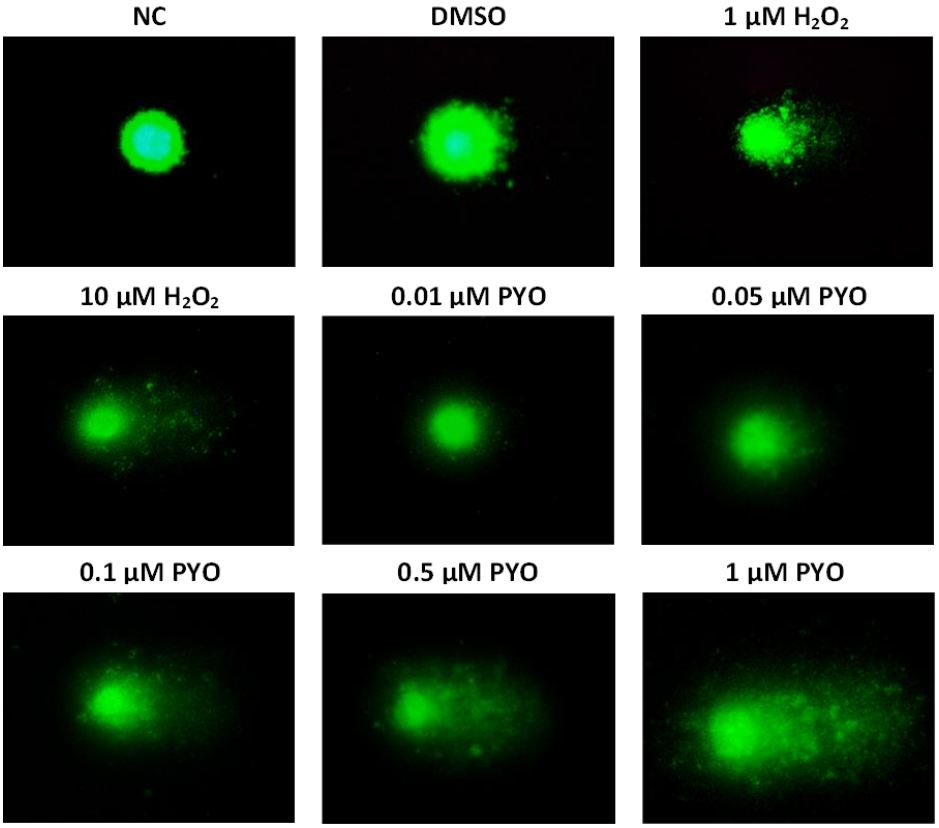

Figure 9 Visualisation of pyocyanin-induced DNA damage in lymphocytes, as detected by the alkaline Comet assay. Lymphocytes were treated with varying concentrations of pyocyanin (0.01 - 1 µM), 1 or 10 µM H_2O_2 (positive controls), DMSO (vehicle control), or left untreated (NC), for 24 hours. DNA damage was assessed via the alkaline Comet assay and fluorescent microscopy was used for visualisation. Images show a representative comet from each treatment group.

3.7 Pyocyanin induces dose-dependent DNA damage in lymphocytes. To evaluate the genotoxic potential of PYO, lymphocytes were exposed to increasing concentrations of PYO (0.01 – 1 µM) for 24 hours and DNA damage was assessed by the alkaline Comet assay.

As shown in Figure 8, treatment with PYO resulted in dose-dependent DNA damage in lymphocytes. Figure 8a shows an increase in mean OTM as PYO concentrations increased. The mean OTM for untreated lymphocytes was 4.59, confirming minimal baseline DNA damage. The OTM increased to 18.12 and 29.98 for 1 and 10 µM H_2O_2 respectively, validating the assay's sensitivity to DNA damage.

All PYO concentrations, excluding 0.05 µM, showed a significant increase in OTM. The lowest concentration of PYO, 0.01 µM, produced a modest 1.7-fold increase in OTM compared to the NC ($p<0.0001$). The OTM continued to increase, producing a substantial increase in DNA damage at 0.5 and 1 µM PYO, with a 7.4 and 7.9-fold increase, respectively, when compared to the NC ($p<0.0001$). This trend was similarly observed in the Tail DNA % (Figure 8b). Tail DNA % quantifies the proportion of DNA which has migrated out of the head, allowing a direct estimate of strand break accumulation. The NC provided a baseline of 3.32% DNA in the comet tails, and this increased to 21.83 and 31.33% for 1 and 10 µM H_2O_2. Similar to the OTM results, 0.01 µM PYO showed a slight increase in Tail DNA % (2.4-fold increase when compared to the NC, $p<0.001$). There was a significant increase in the Tail DNA % for 0.05 µM PYO, not seen in the OTM results ($p<0.01$). The highest concentrations of PYO (0.5 and 1 µM) produced a 9.6 and 10.3-fold increase in Tail DNA % when compared to the NC ($p<0.0001$). Notably, the OTM and Tail DNA % values for 0.5 and 1 µM PYO were comparable to 10 H2O2, if not somewhat greater.

Representative comet images for each treatment condition are shown in Figure 9. These images qualitatively reflect the observed PYO dose-dependent increase in DNA damage seen in Figures 8a and b, indicated by increased tail length and intensity and decreased head intensity in treated cells when compared to the NC.

4.0 Discussion

Pyocyanin (PYO), a vital virulence factor secreted by *Pseudomonas aeruginosa*, has garnered increasing attention for its multifaceted roles in microbial pathogenesis and its potential impact on human health (Shouman et al. 2023; Marey et al. 2024). Its contribution to the establishment and persistence of *P. aeruginosa* has been widely documented, and its redox abilities have been well-studied, but there is a lack of research investigating the genotoxic and cytotoxic potential of this well-known virulence factor (Lau et al. 2004; Muller 2006). This study aimed to elucidate three key aspects of PYO's impact on eukaryotic cells: determine the *in vitro* cytotoxic threshold of PYO in eukaryotic cells, using human lymphocytes as a surrogate; investigate whether and how PYO induces DNA damage in eukaryotic cells; to assess the dose-dependent effects of PYO treatment on both cell viability and DNA damage. Understanding PYO's genotoxic and cytotoxic effects on eukaryotic cells is crucial for assessing the long-term consequences of PYO exposure, especially in patients suffering chronic *P. aeruginosa* infections.

4.1 Biphasic Dose-Response

The CCK-8 assay relies on the reduction of the water-soluble tetrazolium salt WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) by cellular dehydrogenases, primarily mitochondrial enzymes, to produce a yellow-orange formazan dye. The amount of formazan produced is directly proportional to the number of living cells and, when measured via absorbance, provides a quantitative measure of cell viability (Fan et al. 2024). In this study, the CCK-8 assay provided evidence for a biphasic dose-response relationship between PYO and cell survival. Figure 2 illustrates that there are two distinct phases that characterise this biphasic response: hormesis at low concentrations and cytotoxicity at high concentrations.

Cells treated with low concentrations of PYO (0.005 – 0.05 µM) showed an unexpected increase in cell viability after a treatment period of 24 hours. Known as hormesis, this phenomenon describes an effect where a low dose of a potentially harmful agent stimulates a beneficial effect on, in this case, the cell, while a high dose of the same agent would cause harm (Calabrese 2014). This is evident in Figure 2b, whereby, after 48 hours of exposure, treatment with 0.005 and 0.01 µM PYO resulted in a statistically significant increase in cell viability ($p<0.05$) when compared to the negative control (NC). Interestingly, the potential for PYO to induce hormetic effects has been demonstrated in mitochondrial function. A recent study by Peruzzo et al. (2021) found that low concentrations of PYO normalised the membrane potential of mitochondria and mildly increased ROS production and biogenesis in cells with genetic dysfunction in Complex III of the ETC. Although there are no reports on PYO's hormetic effects with regard to cell viability, it is reasonable to suggest that enhanced mitochondrial function at low PYO concentrations could support increased ATP production and metabolic activity, in turn promoting cell survival.

There are likely multiple mechanisms underlying PYO-induced hormesis in lymphocytes. PYO in low concentrations may be able to generate low levels of ROS, which act as cell signalling molecules, triggering adaptive responses to promote cell survival. For example, it is possible that PYO-induced ROS can activate survival pathways, such as the nuclear factor kappa B (NF-κB) (Chai et al. 2014). The NF-κB pathway induces the expression of genes involved in cell survival and anti-apoptotic responses. NF-κB activation leads to the transcription of anti-apoptotic proteins, such as Bcl-2, which prevents mitochondrial permeability transition and cytochrome c release, which is a key trigger for apoptosis (Chen, C., Edelstein and Gelinas, 2000; Kowaltowski, Vercesi and Fiskum, 2000; Eleftheriadis et al., 2016). As Bcl-2 aids in maintaining mitochondrial integrity, the mitochondria continue to produce ATP,

generating a higher absorption value in the CCK-8 assay due to increased formazan production via the cellular dehydrogenases (Dissanayaka et al. 2020). This is a possible explanation for the increased cell viability in cells treated with low concentrations of PYO when compared to the NC; the sublethal levels of ROS in cells treated with low concentrations of PYO delay apoptosis and maintain mitochondrial function via the activation of survival pathways. Therefore, more cells in the low concentration PYO groups remain metabolically active, resulting in enhanced survival when compared to the NC.

Conversely, high concentrations of PYO (1-10 µM) produce the opposite effect in lymphocytes, instead inducing apoptosis. Figure 2 shows an obvious PYO dose-dependent cytotoxic effect at both 24 and 48 hours, with the cytotoxic effects of PYO being especially evident at 5 and 10 µM. At these two concentrations, there was a statistically significant decrease in cell viability after 24 hours ($p<0.001$) and, at 48 hours, both concentrations produced a further decrease in viability. This reduction in cell viability is likely due to overwhelming PYO-induced ROS production. High PYO concentrations may lead to excessive ROS generation (Muller 2002), which exceeds the cell's antioxidant capacity, leading to widespread damage to cellular components. Through this redox activity, PYO directly interferes with mitochondrial function via disruption of the electron transport chain (ETC), causing the collapse of the mitochondrial membrane potential and, consequently, ATP depletion and the release of pro-apoptotic factors (Peruzzo et al. 2021; Managò et al. 2015). Ultimately, this damage leads to the death of the cell, producing an overall decrease in cell viability.

4.3 Time-Dependent Effects on Cell Survival

The comparison of 24- and 48-hour treatments demonstrated a notable time-dependent modulation of PYO's effects on lymphocyte viability, as shown in Figure 3. The increase in cell viability over time suggests a dynamic interaction between PYO-induced stress, cellular mechanisms, and, potentially, adaptive responses. The hormetic response described above was enhanced after a longer exposure to PYO of 48 hours compared to 24 hours across all PYO concentrations and, crucially, there was no increase in viability after 24 hours seen in the control groups (NC, DMSO, 100 µM H_2O_2) or the highest concentration of PYO (10 µM). This time-dependent enhancement of hormesis suggests that the underlying cellular mechanisms are not immediate but rather they involve processes which develop and become more prominent over time.

One possible reason for the increase in cell viability at 48 hours might be the prolonged upregulation of antioxidant defences. Initial exposure to low concentrations of PYO induces an oxidative stress response, potentially triggering the activation of transcription factors such as nuclear factor erythroid 2-related factor (Nrf2) (Xu et al. 2013). In response, Nrf2 binds to antioxidant response elements (AREs) in the promoter regions of genes responsible for producing antioxidant enzymes such as SOD and GPx (Banning et al. 2005). Continued exposure to low concentration PYO might lead to the ongoing activation of Nrf2 and subsequent production of antioxidant enzymes. The Toxic Dose 25 (TD25) results also support this notion, as the TD25 rose over time with cell viability. The continued production of antioxidant enzymes may result in a cumulative increase in antioxidant capacity. Research has shown that the overexpression of peroxiredoxin 5, a cytoprotective antioxidant enzyme capable of neutralising hydrogen peroxide, protected human tendon cells from oxidative stress and subsequent apoptosis (Yuan et al. 2004). PYO-induced production of antioxidant enzymes may confer similar protective advantages to eukaryotic cells, explaining the increase in cell viability seen between 24 and 48 hours.

It is important to note that the increase in cell viability detected by the CCK-8 assay in this study is not likely to be the result of lymphocyte proliferation induced by PYO acting as a mitogen. A review of the

literature provides no evidence to suggest that PYO functions as a mitogen, particularly in lymphocytes, which require highly specific activation signals to undergo proliferation. Instead, the increase in cell viability, particularly between 24 and 48 hours might be more plausibly explained by the stimulation of mitochondrial biogenesis by low concentrations of PYO. Low levels of ROS can act as signalling molecules, stimulating the formation of new mitochondria within the cell (Peruzzo et al. 2021). This process is often mediated by the transcription factor PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) and takes hours or days to complete (Fernandez-Marcos and Auwerx 2011). Since the CCK-8 assay measures mitochondrial dehydrogenase activity, an increase in the number of mitochondria would result in higher measured cell viability. In the NC group, cells remain in a metabolically quiescent state due to the absence of external stimuli, but they retain their ability to respond to signals when activated. The introduction of PYO may act as a stimulus, triggering activation of the cells and increasing metabolic activity in the mitochondria and the generation of new mitochondria over time. When this is assessed with the CCK-8 assay it translates to an increase in cell viability.

4.4 DNA Damage and Pyocyanin Intercalation

Despite the clear dose-dependent cytotoxicity observed at high PYO concentrations in the CCK-8 assay, the PicoGreen assay showed no evidence of a similar dose-dependent relationship between PYO concentration and SSBs. The PicoGreen assay is based on the ability of the fluorescent dye, PicoGreen, to bind to double-stranded DNA (dsDNA), making it possible to directly measure the denaturation of dsDNA (Dragan et al. 2010). Hydrogen bonds in the DNA double helix are destabilised in alkaline solutions (pH 12), and the presence of SSBs and alkali-labile sites in the DNA affects the kinetics of the unwinding of the dsDNA. When SSBs and alkali-labile sites are present, the DNA unwinds faster than undamaged DNA. As such, the use of PicoGreen allows the remaining dsDNA to be quantified to determine the frequency of SSBs in the DNA.

Following the principle of the PicoGreen assay, the %dsDNA should decrease faster as DNA damage increases, however, Figure 5 shows that this did not occur. Figure 5 shows the %dsDNA remaining in lymphocytes treated with varying concentrations of PYO over a 30-minute period of alkaline unwinding. Interestingly, the graph shows an immediate drop in %dsDNA at t=0 for all PYO treated groups, except the 0.5 µM group. This sudden decrease in %dsDNA is not dose-dependent, meaning that the decrease is similar across the PYO treated groups (not 0.5 µM) regardless of concentration. If the cause of this initial decrease were due to the presence of SSBs, a clear dose-dependent relationship would be expected with higher PYO concentrations showing a greater initial decrease in %dsDNA.

Furthermore, PYO treated groups did not show an expected rapid decrease in dsDNA% over the 30-minute unwinding period. The kinetics of unwinding dsDNA should increase with increasing DNA damage, evidenced by the 50 µM H_2O_2 treated group in Figure 5, where the %dsDNA decreased from 85% to 44%. Comparing this to the highest concentration of PYO, 1 µM, where the %dsDNA decreased from 86% to 77% over the 30-minute period, shows that there was no notable change in the DNA unwinding kinetics for the PYO treated group. The lowest concentration of PYO, 0.005 µM, did demonstrate increased unwinding kinetics, however this was not significant. Overall, the lack of a dose-dependent decrease in %dsDNA over the unwinding period, coupled with the slow kinetics of the unwinding dsDNA in PYO treated cells suggests that PYO might have the potential to intercalate with DNA.

PYO intercalation with DNA is not well-studied, but the PicoGreen assay results in this study are supported by the research of Das et al. (2015). It was found that PYO could displace ethidium bromide (EtBr), a well-known intercalating agent, bound to dsDNA. This displacement of EtBr suggests that PYO,

too, is able to intercalate with DNA and might compete with other intercalating agents to do so. The lowest concentration of PYO in this study showed more typical kinetics of DNA unwinding when compared to the other PYO treated groups. At low concentrations, there is less PYO available to intercalate into the DNA, allowing denaturation to proceed more efficiently, therefore resulting in a more typical reduction of %dsDNA over time. This is further evidenced by the SSF x (-1) values in Figure 6, whereby the lowest PYO concentration produced the highest SSF x (-1) value, indicating that this treatment group had the highest level of DNA damage. As the PYO concentrations increase, intercalation becomes more pronounced, impeding strand separation and producing consistently high %dsDNA measurements, regardless of the actual DNA damage.

4.5 Genotoxicity and the Comet Assay

The alkaline Comet assay allowed further investigation into the potential genotoxic effects of PYO in lymphocytes. The Comet assay has emerged as one of the most sensitive and versatile methods for detecting DNA damage at the level of the individual cell (McKelvey-Martin et al. 1993). Under alkaline conditions (pH>13), the assay is particularly sensitive to SSBs and alkali-labile sites. During electrophoresis, fragmented DNA migrates towards the anode, forming the “comet” pattern that is visualised via fluorescent microscopy. Comet heads are comprised of intact DNA, while tails contain the broken DNA fragments. The extent of the DNA damage can be quantified using parameters such as Olive Tail Moment (OTM) and Tail DNA % (TDNA%), which were used in this study. The sensitivity of the Comet assay allows for the detection of DNA damage induced by very low concentrations of genotoxic agents which exert subtle but significant effects.

The results in Figure 8 demonstrate a statistically significant increase in DNA damage in lymphocytes following exposure to increasing concentrations of PYO for 24 hours. Both OTM and TDNA% values increased progressively with increasing PYO concentration. For example, treatment with 0.01 µM PYO resulted in a slight, but significant, increase in both OTM and TDNA% when compared to the NC. Exposure to the highest concentration of PYO yielded substantially greater values for both measurements, showing a clear dose-dependent relationship between DNA damage and PYO concentration. While there is no literature which directly supports the dose-dependent PYO-induced DNA damage in eukaryotic cells, research by Priyaja et al. (2014) found that, in human embryonic lung cells, PYO exposure led to the production of hydrogen peroxide in a dose-dependent manner, with 119 µM PYO producing a 53% increase in hydrogen peroxide. Although the study did not directly measure DNA damage in human lung embryonic cells, hydrogen peroxide is a well-known genotoxin capable of inducing oxidative DNA damage via ROS generation and is produced as a consequence of PYO’s redox cycling. These findings, coupled with the dose-dependent PYO-induced DNA damage determined by the Comet assay, suggest that PYO exposure induces genotoxicity in lymphocytes, likely through mechanisms of oxidative stress.

Interestingly, a comparison of the two parameters measured shows that there are relatively small differences between the mean OTM and TDNA% values across all treatment groups. This suggests that the extent of DNA migration is largely proportional to the overall percentage of fragmented DNA, implying that PYO-induced DNA damage results in relatively uniform fragmentation rather than producing small, widely dispersed fragments, which would indicate a majority of SSBs (Olive, Banáth and Fjell, 1994). This observation is consistent with previous studies that have identified a strong correlation between OTM and TDNA% in models of oxidative DNA damage. (A. Čabarkapa et al., 2014).

The similarity between these two measurements reinforces the notion that PYO induces genotoxicity via oxidative mechanisms, leading to consistent patterns of DNA strand breakage.

Importantly, the DNA damage demonstrated by the alkaline Comet assay, when combined with the PicoGreen assay results, further supports the hypothesis of PYO intercalation with DNA. The observation of substantial DNA damage in the Comet assay alongside the anomalous PicoGreen assay results strengthens the hypothesis that the PicoGreen assay's inability to detect increased SSBs was not an actual absence of DNA damage, but rather the interference of PYO intercalation. The DNA damage caused by PYO intercalation could have severe consequences, inducing local structural changes to the DNA, which can impede the progression of DNA polymerase during replication, leading to incomplete DNA synthesis (Sheaff, Ilsley and Kuchta 1991). Intercalators can also interfere with the recognition and binding of DNA repair enzymes, compromising the cells' ability to correct other forms of DNA damage, including that caused by PYO's redox activity (Zhou, J. et al. 2024). The potential combined disruption of replication, transcription, and repair caused by PYO intercalation could have severe consequences for patients suffering from chronic *P. aeruginosa* infections, as they can lead to an accumulation of mutations, potentially triggering apoptosis or contributing to uncontrolled cell proliferation, a hallmark of cancer (Espinoza et al. 2024; Kloeber and Lou 2021).

The combined DNA damage induced by PYO's oxidative and intercalative effects presents clinical concerns for patients suffering from chronic *P. aeruginosa* infections. Continued oxidative stress can result in excessive DNA damage, leading to the accumulation of mutations if not repaired correctly. Over time, this genomic instability and mutation accumulation may lead to an increased risk of malignant transformation in the affected cells. Patients suffering chronic infections, such as those with CF, are likely to be exposed to pyocyanin for long periods, and this compounding DNA damage might elevate the risk of oncogenesis. While there have been no investigations into whether PYO does increase the risk of cancer, some studies have found a link between bacterial infection-induced oxidative stress as a result of chronic inflammation. For example, chronic *Helicobacter pylori* infections have been found to increase the risk of gastric carcinoma (Parsonnet et al. 1991). PYO is known for contributing to chronic inflammation in *P. aeruginosa* infections, a process which is often associated with the development and progression of cancer (Denning et al. 1998; Karin 2006).

4.6 Limitations and Future Work

The *in vitro* design of this study was a significant limitation, as it lacked variables seen in hosts, such as immune systems and the complex microenvironments bacteria encounter within a living organism (Crabbé, Ledesma and Nickerson 2014). In order to improve the translatability of results, future research should prioritise the use of *in vivo* models, such as animal studies, to gain more understanding of PYO's effects on host cells within the context of a *P. aeruginosa* infection.

With regard to the results of this study, future research should further investigate the specific molecular signalling pathways activated during PYO-induced hormesis, with particular focus on how this process promotes cell survival at low doses. PYO-induced hormesis may be tied to stress-response mechanisms, including the upregulation of antioxidant defences and mitochondrial biogenesis (López-Martínez and Hahn 2012; Palmeira et al. 2019). Understanding the underlying mechanisms of this hormetic response could lead to the identification of novel therapeutic strategies to target these specific pathways, such as the Nrf2 pathway, for protective effects against PYO-induced oxidative damage.

Moreover, PYO's potential to intercalate with DNA is an area that requires further investigation, especially concerning the possible subsequent DNA damage. Other intercalating agents have been shown to contribute towards malignancies (Agudelo et al., 2014) and future work is necessary to fully understand PYO's intercalating abilities and its potential to cause malignancies, especially in patients with chronic exposure. Fluorescence resonance energy transfer (FRET) assays are a potential way to gain insight into the specific mechanisms of PYO intercalation abilities (Didenko, 2001). This technique is able to detect molecular interactions and, by assessing changes in FRET efficiency upon PYO binding, allows the determination of the mechanisms and structural distortions of PYO intercalation. This would allow for a clearer understanding of PYO-induced genotoxicity and DNA damage, contributing to the broader understanding of PYO's role in bacterial pathogenesis.

Future studies should look into whether long-term exposure to PYO leads to the accumulation of DNA damage over time. One limitation of this study is the use of short treatment periods and lack of diverse cell types. Due to these limitations, the results do not reflect the long-term effects of PYO exposure or represent the diverse cells and tissues affected by *P. aeruginosa*. As such, future work should investigate the long-term effects of PYO exposure on various cell types in order to reveal whether certain cell types are more prone to genotoxicity or cellular dysfunction. Future studies should also aim to investigate if this genotoxicity has led to greater consequences for patients with P. aeruginosa infections, such as an unestablished link between chronic infections with this pathogen and tumour formation.

4.7 Conclusion

To conclude, this study highlights the complex effects of pyocyanin on host cells. The findings from this study indicate that, while PYO induces genotoxicity, it may also trigger adaptive cellular responses to produce a hormetic effect at low doses. More research is needed to elucidate the exact mechanisms underlying these effects. Due to the limitations of this study, particularly its *in vitro* nature, future research should focus on validating these findings in physiologically relevant models, including *in vivo* systems. Additionally, further investigation into PYO's potential to intercalate with DNA should be conducted via methodologies like FRET assays. Long-term studies assessing the cumulative effects of PYO exposure are also critical to understand PYO's full impact on host cells. By addressing these gaps, future research can provide further insight into the effects of PYO on host cells, with particular attention to its genotoxic potential and its wider implications for the outcomes of patients suffering chronic *P. aeruginosa* infections.

References

Abdelaziz, A., Abo, A.M., Al-Monofy, K.B. and Al-Madboly, L.A. (2023) Pseudomonas Aeruginosa's greenish-blue Pigment pyocyanin: its Production and biological activities. *Microbial Cell Factories*, 22 (1).

Agudelo, D., Bourassa, P., Bérubé, G. and Tajmir-Riahi, H.-A. (2014) Intercalation of antitumor drug doxorubicin and its analogue by DNA duplex: Structural features and biological implications. *International Journal of Biological Macromolecules*, 66, pp. 144–150.

Al-Shabib, N.A., Husain, F.M., Khan, R.A., Khan, M.S., Alam, M.Z., Ansari, F.A., Laeeq, S., Zubair, M., Shahzad, S.A., Khan, J.M., Alsalme, A. and Ahmad, I. (2019) Interference of phosphane copper (I) complexes of β-carboline with quorum sensing regulated virulence functions and biofilm in foodborne pathogenic bacteria: A first report. *Saudi Journal of Biological Sciences*, 26 (2), pp. 308–316.

Banning, A., Deubel, S., Kluth, D., Zhou, Z. and Brigelius-Flohé, R. (2005) The GI-GPx gene is a target for Nrf2. *Molecular and Cellular Biology* [Post-print], 25 (12), pp. 4914–4923. Available from https://pubmed.ncbi.nlm.nih.gov/15923610/.

Barakat, R., Goubet, I., Manon, S., Berges, T. and Rosenfeld, E. (2013) Unsuspected pyocyanin effect in yeast under anaerobiosis. *MicrobiologyOpen*, 3 (1), pp. 1–14.

Blomquist, K.C. and Nix, D.E. (2020) A Critical Evaluation of Newer β-Lactam Antibiotics for Treatment of Pseudomonas aeruginosa Infections. *Annals of Pharmacotherapy*, 55 (8), p. 106002802097400.

Burssed, B., Zamariolli, M., Bellucco, F.T. and Melaragno, M.I. (2022) Mechanisms of structural chromosomal rearrangement formation. *Molecular Cytogenetics*, 15 (1).

Cabarkapa, A., Zivković, L., Zukovec, D., Djelić, N., Bajić, V., Dekanski, D., Spremo-Potparević, B. (2014) Protective effect of dry olive leaf extract in adrenaline induced DNA damage evaluated using in vitro comet assay with human peripheral leukocytes. Toxicology in Vitro [Post-print], 28 (3), pp. 451–456. Available from https://www.researchgate.net/publication/267033616_Protective_effect_of_dry_olive_leaf_extract_in_adrenaline_induced_DNA_damage_evaluated_using_in_vitro_comet_assay_with_human_peripheral_leukocytes.

Calabrese, E. (2014) Hormesis: a fundamental concept in biology. *Microbial Cell*, 1 (5), pp. 145–149.

Chai, W., Zhang, J., Duan, Y., Pan, D., Liu, W., Li, Y., Yan, X. and Chen, B. (2014) Pseudomonas pyocyanin stimulates IL-8 expression through MAPK and NF-κB pathways in differentiated U937 cells. *BMC Microbiology* [Post-print], 14 (1), p. 26. Available from https://pmc.ncbi.nlm.nih.gov/articles/PMC3925954/.

Chen, C., Edelstein, L.C. and Gelinas, C. (2000) The Rel/NF-kappa B Family Directly Activates Expression of the Apoptosis Inhibitor Bcl-xL. *Molecular and Cellular Biology*, 20 (8), pp. 2687–2695.

Crabbé, A., Ledesma, M.A. and Nickerson, C.A. (2014) Mimicking the host and its microenvironmentin vitrofor studying mucosal infections byPseudomonas aeruginosa. *Pathogens and Disease*, 71 (1), pp. 1–19.

Das, T., Kutty, S.K., Tavallaie, R., Ibugo, A.I., Panchompoo, J., Sehar, S., Aldous, L., Yeung, A.W.S., Thomas, S.R., Kumar, N., Gooding, J.J. and Manefield, M. (2015) Phenazine virulence factor binding to extracellular DNA is important for Pseudomonas aeruginosa biofilm formation. *Scientific Reports*, 5 (1).

Das, T. and Manefield, M. (2012) Pyocyanin Promotes Extracellular DNA Release in Pseudomonas aeruginosa. *PLoS ONE*, 7 (10), p. e46718.

Davis, G. and Thornalley, P.J. (1983) Free radical production from the aerobic oxidation of reduced pyridine nucleotides catalysed by phenazine derivatives. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* [Post-print], 724 (3), pp. 456–464. Available from https://www.sciencedirect.com/science/article/abs/pii/0005272883901068.

Denning, G.M., Wollenweber, L.A., Railsback, M.A., Cox, C.D., Stoll, L.L. and Britigan, B.E. (1998) *Pseudomonas*Pyocyanin Increases Interleukin-8 Expression by Human Airway Epithelial Cells. *Infection and Immunity*, 66 (12), pp. 5777–5784.

Didenko, V.V. (2001) DNA Probes Using Fluorescence Resonance Energy Transfer (FRET): Designs and Applications. *BioTechniques*, 31 (5), pp. 1106–1121.

Dissanayaka, W.L., Han, Y., Zhang, L., Zou, T. and Zhang, C. (2020) Bcl-2 Overexpression and Hypoxia Synergistically Enhance Angiogenic Properties of Dental Pulp Stem Cells. *International Journal of Molecular Sciences* [Post-print], 21 (17), p. 6159. Available from https://www.mdpi.com/1422-0067/21/17/6159.

Dragan, A.I., Casas-Finet, J.R., Bishop, E.S., Strouse, R.J., Schenerman, M.A. and Geddes, C.D. (2010) Characterization of PicoGreen Interaction with dsDNA and the Origin of Its Fluorescence Enhancement upon Binding. *Biophysical Journal* [Post-print], 99 (9), pp. 3010–3019. Available from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2965993/.

Driessens, N., Versteyhe, S., Ghaddhab, C., Burniat, A., De Deken, X., Van Sande, J., Dumont, J.-E., Miot, F. and Corvilain, B. (2009) Hydrogen peroxide induces DNA single- and double-strand breaks in thyroid cells and is therefore a potential mutagen for this organ. *Endocrine-related cancer* [Post-print], 16 (3), pp. 845–56. Available from https://www.ncbi.nlm.nih.gov/pubmed/19509065.

El-Fouly, M.Z., Sharaf, A.M., Shahin, A.A.M., El-Bialy, H.A. and Omara, A.M.A. (2015) Biosynthesis of pyocyanin pigment by Pseudomonas aeruginosa. *Journal of Radiation Research and Applied Sciences*, 8 (1), pp. 36–48.

Eleftheriadis, T., Pissas, G., Liakopoulos, V. and Stefanidis, I. (2016) Cytochrome c as a Potentially Clinical Useful Marker of Mitochondrial and Cellular Damage. *Frontiers in Immunology*, 7.

Espinoza, J.A., Kanellis, D.C., Sheetanshu Saproo, Leal, K., Martinez, J., Bartek, J. and Mikael S Lindström (2024) Chromatin damage generated by DNA intercalators leads to degradation of RNA Polymerase II. *Nucleic Acids Research*, 52 (8).

Fan, J., Schiemer, T., Vaska, A., Jahed, V. and Klavins, K. (2024) Cell via Cell Viability Assay Changes Cellular Metabolic Characteristics by Intervening with Glycolysis and Pentose Phosphate Pathway. *Chemical Research in Toxicology* [Post-print], 37 (2). Available from https://pubmed.ncbi.nlm.nih.gov/38191130/.

Fernanda Jiménez Otero, Newman, D.K. and Tender, L.M. (2023) Pyocyanin-dependent electrochemical inhibition of *Pseudomonas aeruginosa* biofilms is synergistic with antibiotic treatment. *MBio* [Post-print], 14 (4). Available from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10470778/.

Fernandez-Marcos, P.J. and Auwerx, J. (2011) Regulation of PGC-1α, a nodal regulator of mitochondrial biogenesis. *The American Journal of Clinical Nutrition*, 93 (4), pp. 884S890S.

Fleming, A.M. and Burrows, C.J. (2021) Chemistry of ROS-mediated oxidation to the guanine base in DNA and its biological consequences. *International Journal of Radiation Biology*, 98 (3), pp. 1–9.

Garcia-Nuñez, M., Marti, S., Puig, C., Perez-Brocal, V., Millares, L., Santos, S., Ardanuy, C., Moya, A., Liñares, J. and Monsó, E. (2017) Bronchial microbiome, PA biofilm-forming capacity and exacerbation in severe COPD patients colonized by P. aeruginosa. *Future Microbiology* [Post-print], 12 (22), pp. 379–392. Available from https://pubmed.ncbi.nlm.nih.gov/28339291/.

Grosso-Becerra, M.V., Croda-García, G., Merino, E., Servín-González, L., Mojica-Espinosa, R. and Soberón-Chávez, G. (2014) Regulation of Pseudomonas aeruginosa virulence factors by two novel RNA thermometers. *Proceedings of the National Academy of Sciences of the United States of America* [Post-print], 111 (43), pp. 15562–15567. Available from https://pubmed.ncbi.nlm.nih.gov/25313031/.

Henderson, L., Wolfreys, A., Fedyk, J., Bourner, C. and Windebank, S. (1998) The ability of the Comet assay to discriminate between genotoxins and cytotoxins. *Mutagenesis*, 13 (1), pp. 89–94.

Jefferies, K.C., Cipriano, D.J. and Forgac, M. (2008) Function, structure and regulation of the vacuolar (H+)-ATPases. *Archives of Biochemistry and Biophysics*, 476 (1), pp. 33–42.

Karin, M. (2006) Nuclear factor-κB in cancer development and progression. *Nature*, 441 (7092), pp. 431–436.

Kloeber, J.A. and Lou, Z. (2021) Critical DNA damaging pathways in tumorigenesis. *Seminars in Cancer Biology*, 85.

Koley, D., Ramsey, M.M., Bard, A.J. and Whiteley, M. (2011) Discovery of a biofilm electrocline using real-time 3D metabolite analysis. *Proceedings of the National Academy of Sciences*, 108 (50), pp. 19996–20001.

Kowaltowski, A.J., Vercesi, A.E. and Fiskum, G. (2000) Bcl-2 prevents mitochondrial permeability transition and cytochrome c release via maintenance of reduced pyridine nucleotides. *Cell Death & Differentiation*, 7 (10), pp. 903–910.

Kuzminov, A. (2001) Single-strand interruptions in replicating chromosomes cause double-strand breaks. *Proceedings of the National Academy of Sciences*, 98 (15), pp. 8241–8246.

Lansbury, L., Lim, B., Baskaran, V. and Lim, W.S. (2020) Co-infections in people with COVID-19: a systematic review and meta-analysis. *The Journal of Infection* [Post-print], 81 (2), pp. 266–275. Available from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7255350/.

Lau, G.W., Hassett, D.J., Ran, H. and Kong, F. (2004) The role of pyocyanin in Pseudomonas aeruginosa infection. *Trends in Molecular Medicine*, 10 (12), pp. 599–606.

Lauredo, I.T., Sabater, J.R., Ahmed, A., Botvinnikova, Y. and Abraham, W.M. (1998) Mechanism of pyocyanin- and 1-hydroxyphenazine-induced lung neutrophilia in sheep airways. *Journal of Applied Physiology*, 85 (6), pp. 2298–2304.

Lebeaux, D., Chauhan, A., Rendueles, O. and Beloin, C. (2013) From in vitro to in vivo Models of Bacterial Biofilm-Related Infections. *Pathogens*, 2 (2), pp. 288–356.

Li, Z. and Nair, S.K. (2012) Quorum sensing: How bacteria can coordinate activity and synchronize their response to external signals? *Protein Science* [Post-print], 21 (10), pp. 1403–1417. Available from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3526984/.

López-Martínez, G. and Hahn, D.A. (2012) Short-term anoxic conditioning hormesis boosts antioxidant defenses, lowers oxidative damage following irradiation and enhances male sexual performance in the Caribbean fruit fly, *Anastrepha suspensa*. *Journal of Experimental Biology*, 215 (12), pp. 2150–2161.

Managò, A., Becker, K.A., Carpinteiro, A., Wilker, B., Soddemann, M., Seitz, A.P., Edwards, M.J., Grassmé, H., Szabò, I. and Gulbins, E. (2015) Pseudomonas aeruginosaPyocyanin Induces Neutrophil DeathviaMitochondrial Reactive Oxygen Species and Mitochondrial Acid Sphingomyelinase. *Antioxidants & Redox Signaling*, 22 (13), pp. 1097–1110.

Marey, M.A., Abozahra, R., El-Nikhely, N.A., Kamal, M.F., Abdelhamid, S.M. and El-Kholy, M.A. (2024) Transforming microbial pigment into therapeutic revelation: extraction and characterization of pyocyanin from Pseudomonas aeruginosa and its therapeutic potential as an antibacterial and anticancer agent. *Microbial Cell Factories*, 23 (1).

Mavrodi, D.V., Bonsall, R.F., Delaney, S.M., Soule, M.J., Phillips, G. and Thomashow, L.S. (2001) Functional Analysis of Genes for Biosynthesis of Pyocyanin and Phenazine-1-Carboxamide from Pseudomonas aeruginosa PAO1. *Journal of Bacteriology* [Post-print], 183 (21), pp. 6454–6465. Available from https://jb.asm.org/content/jb/183/21/6454.full.pdf.

McDonald, R.J., Pan, L.C., St George, J.A., Hyde, D.M. and Ducore, J.M. (1993) Hydrogen peroxide induces DNA single strand breaks in respiratory epithelial cells. *Inflammation* [Post-print], 17 (6), pp. 715–22. Available from https://pubmed.ncbi.nlm.nih.gov/8112830/.

McKelvey-Martin, V.J., Green, M.H.L., Schmezer, P., Pool-Zobel, B.L., De Méo, M.P. and Collins, A. (1993) The single cell gel electrophoresis assay (comet assay): A European review. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 288 (1), pp. 47–63.

Mills, G.C. (2004) Glutathione peroxidase and the destruction of hydrogen peroxide in animal tissues. *Archives of Biochemistry and Biophysics* [Post-print], 86 (1), pp. 1–5. Available from https://www.sciencedirect.com/science/article/abs/pii/000398616090357X.

Muller, M. (2002) Pyocyanin induces oxidative stress in human endothelial cells and modulates the glutathione redox cycle. *Free Radical Biology and Medicine*, 33 (11), pp. 1527–1533.

Muller, M. (2006) Premature cellular senescence induced by pyocyanin, a redox-active Pseudomonas aeruginosa toxin. *Free Radical Biology and Medicine*, 41 (11), pp. 1670–1677.

Muller, M. (2011) Glutathione modulates the toxicity of, but is not a biologically relevant reductant for, the Pseudomonas aeruginosa redox toxin pyocyanin. *Free Radical Biology and Medicine*, 50 (8), pp. 971–977.

Muller, M., Li, Z. and Maitz, P.K.M. (2009) Pseudomonas pyocyanin inhibits wound repair by inducing premature cellular senescence: Role for p38 mitogen-activated protein kinase. *Burns* [Post-print], 35 (4), pp. 500–508. Available from https://www.sciencedirect.com/science/article/pii/S0305417908003598?via%3Dihub.

O'Connor, Mark J. (2015) Targeting the DNA Damage Response in Cancer. *Molecular Cell*, 60 (4), pp. 547–560.

O'Malley, Y.Q., Abdalla, M.Y., McCormick, M.L., Reszka, K.J., Denning, G.M. and Britigan, B.E. (2003) Subcellular localization of Pseudomonas pyocyanin cytotoxicity in human lung epithelial cells. *American Journal of Physiology. Lung Cellular and Molecular Physiology* [Post-print], 284 (2), pp. L420-430. Available from https://pubmed.ncbi.nlm.nih.gov/12414438/.

O'Malley, Y.Q., Reszka, K.J., Spitz, D.R., Denning, G.M. and Britigan, B.E. (2004) Pseudomonas aeruginosa pyocyanin directly oxidizes glutathione and decreases its levels in airway epithelial cells. *American Journal of Physiology. Lung Cellular and Molecular Physiology* [Post-print], 287 (1), pp. L94-103. Available from https://pubmed.ncbi.nlm.nih.gov/15020296/.

Okshevsky, M. and Meyer, R.L. (2013) The role of extracellular DNA in the establishment, maintenance and perpetuation of bacterial biofilms. *Critical Reviews in Microbiology*, 41 (3), pp. 341–352.

Olive, P.L., Banáth, J.P. and Fjell, C.D. (1994) DNA strand breakage and DNA structure influence staining with propidium iodide using the alkaline comet assay. *Cytometry*, 16 (4), pp. 305–312.

Oliver, A. (2000) High Frequency of Hypermutable Pseudomonas aeruginosa in Cystic Fibrosis Lung Infection. *Science*, 288 (5469), pp. 1251–1253.

Palmeira, C.M., Teodoro, J.S., Amorim, J.A., Steegborn, C., Sinclair, D.A. and Rolo, A.P. (2019) Mitohormesis and metabolic health: The interplay between ROS, cAMP and sirtuins. *Free radical biology & medicine* [Post-print], 141, pp. 483–491. Available from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6718302/.

Pan, J., Chang, Q., Wang, X., Son, Y., Zhang, Z., Chen, G., Luo, J., Bi, Y., Chen, F. and Shi, X. (2010) Reactive Oxygen Species-Activated Akt/ASK1/p38 Signaling Pathway in Nickel Compound-Induced Apoptosis in BEAS 2B Cells. *Chemical Research in Toxicology*, 23 (3), pp. 568–577.

Paprocka, P., Durnaś, B., Mańkowska, A., Król, G., Wollny, T. and Bucki, R. (2022) Pseudomonas aeruginosa Infections in Cancer Patients. *Pathogens*, 11 (6), p. 679.

Parsonnet, J., Friedman, G.D., Vandersteen, D.P., Chang, Y., Vogelman, J.H., Orentreich, N. and Sibley, R.K. (1991) Helicobacter pyloriInfection and the Risk of Gastric Carcinoma. *New England Journal of Medicine*, 325 (16), pp. 1127–1131.

Peruzzo, R., Corrà, S., Costa, R., Brischigliaro, M., Varanita, T., Biasutto, L., Rampazzo, C., Ghezzi, D., Leanza, L., Zoratti, M., Zeviani, M., De Pittà, C., Viscomi, C., Costa, R. and Szabò, I. (2021) Exploiting pyocyanin to treat mitochondrial disease due to respiratory complex III dysfunction. *Nature Communications* [Post-print], 12 (1). Available from https://www.nature.com/articles/s41467-021-22062-x.

Priyaja, P., Jayesh, P., Philip, R. and Bright Singh, I.S. (2014) Pyocyanin induced in vitro oxidative damage and its toxicity level in human, fish and insect cell lines for its selective biological applications. *Cytotechnology*, 68 (1), pp. 143–155.

Qiu, Z., Zhang, Z., Roschke, A., Varga, T. and Aplan, P.D. (2017) Generation of Gross Chromosomal Rearrangements by a Single Engineered DNA Double Strand Break. *Scientific Reports* [Post-print], 7 (1), p. 43156. Available from https://www.nature.com/articles/srep43156#:~:text=Gross%20chromosomal%20rearrangements%20(GCRs)%2C.

Quinton, P.M. (2008) Cystic fibrosis: impaired bicarbonate secretion and mucoviscidosis. *Lancet* [Post-print], 372 (9636), pp. 415–417. Available from https://escholarship.org/uc/item/0hg0w4dv.

Rada, B., Lekstrom, K., Damian, S., Dupuy, C. and Leto, T.L. (2008) The *Pseudomonas* Toxin Pyocyanin Inhibits the Dual Oxidase-Based Antimicrobial System as It Imposes Oxidative Stress on Airway Epithelial Cells. *Journal of Immunology*, 181 (7), pp. 4883–4893.

Ran, H., Hassett, D.J. and Lau, G.W. (2003) Human targets of Pseudomonas aeruginosa pyocyanin. *Proceedings of the National Academy of Sciences of the United States of America* [Post-print], 100 (24), pp. 14315–14320. Available from https://pubmed.ncbi.nlm.nih.gov/14605211/.

Reid, D.M., Anderson, G.J. and Lamont, I.L. (2008) Cystic fibrosis: ironing out the problem of infection? *Am J Physiol Lung Cell Mol Physiol.*, 295 (1), pp. L23–L24.

Schröder, H.C., Batel, R., Schwertner, H., Boreiko, O. and Müller, W.E.G. (2006) Fast micromethod DNA single-strand-break assay. *Methods in molecular biology (Clifton, N.J.)* [Post-print], 314, pp. 287–305. Available from https://pubmed.ncbi.nlm.nih.gov/16673889/.

Sheaff, R., Ilsley, D. and Kuchta, R. (1991) Mechanism of DNA polymerase .alpha. inhibition by aphidicolin. *Biochemistry*, 30 (35), pp. 8590–8597.

Shibutani, S., Takeshita, M. and Grollman, A.P. (1991) Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. *Nature* [Post-print], 349 (6308), pp. 431–434. Available from https://www.nature.com/articles/349431a0.

Shouman, H., Heba Shehta Said, Kenawy, H.I. and Hassan, R. (2023) Molecular and biological characterization of pyocyanin from clinical and environmental Pseudomonas aeruginosa. *Microbial Cell Factories*, 22 (1).

Stewart-Tull, D.E. and Armstrong, A.V. (1972) The effect of 1-hydroxyphenazine and pyocyanin from Pseudomonas aeruginosa on mammalian cell respiration. *Journal of medical microbiology* [Post-print], 5 (1), pp. 67–73. Available from https://pubmed.ncbi.nlm.nih.gov/4623349/.

Usher, L.R., Lawson, R.A., Geary, I., Taylor, C.J., Bingle, C.D., Taylor, G.W. and Whyte, M.K.B. (2002) Induction of Neutrophil Apoptosis by thePseudomonas aeruginosaExotoxin Pyocyanin: A Potential Mechanism of Persistent Infection. *The Journal of Immunology*, 168 (4), pp. 1861–1868.

Van Laar, T.A., Esani, S., Birges, T.J., Hazen, B., Thomas, J.M. and Rawat, M. (2018) Pseudomonas aeruginosa *gshA* Mutant Is Defective in Biofilm Formation, Swarming, and Pyocyanin Production. *mSphere*, 3 (2).

Verbon, E.H., Post, J.A. and Boonstra, J. (2012) The influence of reactive oxygen species on cell cycle progression in mammalian cells. *Gene* [Post-print], 511 (1), pp. 1–6. Available from https://pubmed.ncbi.nlm.nih.gov/22981713/.

Vidaillac, C. and Chotirmall, S.H. (2021) Pseudomonas aeruginosa in bronchiectasis: infection, inflammation, and therapies. *Expert Review of Respiratory Medicine*, 15 (5), pp. 649–662.

Xu, Y., Duan, C., Kuang, Z., Hao, Y. and Lau, G.W. (2013) Pseudomonas aeruginosa pyocyanin activates NRF2-ARE-mediated transcriptional response via the ROS-EGFR-PI3K-AKT/MEK-ERK MAP kinase signaling in pulmonary epithelial cells. *PLOS One* [Post-print], 8 (8), p. e72528. Available from https://www.researchgate.net/publication/256470413_Pseudomonas_aeruginosa_pyocyanin_activates_NRF2-ARE-mediated_transcriptional_response_via_the_ROS-EGFR-PI3K-AKTMEK-ERK_MAP_kinase_signaling_in_pulmonary_epithelial_cells.

Yuan, J., Murrell, G.A.C., Trickett, A., Landtmeters, M., Knoops, B. and Wang, M.-X. (2004) Overexpression of antioxidant enzyme peroxiredoxin 5 protects human tendon cells against apoptosis and loss of cellular function during oxidative stress. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1693 (1), pp. 37–45.

Zhou, J., Chen, Q., Ren, R., Yang, J., Liu, B., Horton, J.R., Chang, C., Li, C., Maksoud, L., Yang, Y., Rotili, D., Jain, A.K., Zhang, X., Blumenthal, R.M., Chen, T., Gao, Y., Valente, S., Mai, A. and Cheng, X. (2024) Quinoline-based compounds can inhibit diverse enzymes that act on DNA. *Cell Chemical Biology* [Post-print], 31 (12). Available from https://www.sciencedirect.com/science/article/pii/S2451945624004033.