

**UNIVERSITY OF GHANA
COLLEGE OF BASIC AND APPLIED SCIENCES**

**THE ROLE OF NATURAL ANTIOXIDANTS IN THE ATTENUATION OF
MYCOLACTONE TOXICITY IN BURULI ULCER DISEASE**

BY

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DECLARATION

I YVONNE YAA ASANTEWAA, declare that except for references to the work of others for which I have acknowledged, the experimental work described in this project was performed by me in the Department of Biochemistry, Cell and Molecular Biology under the supervisions of Dr Lydia Mosi, Dr Jonathan P Adjimani and Rev Dr W S K Gbewonyo. Neither all nor parts of this project have been presented for another degree elsewhere.

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ABSTRACT

Mycobacterium ulcerans' mycolactone, is reported to mediate the production of ROS in keratinocytes; cells that play critical role in wound healing. The key stimulatory function of macrophages in the production of keratinocytes and fibroblasts during wound healing may also be impaired by mycolactone-mediated ROS. To ensure regeneration of tissues with proper and timely healing of the wounds, antioxidants that can combat the effects of ROS ought to be investigated. This study was therefore aimed at determining ROS activity in RAW 264.7 macrophages in the presence of mycolactone using fluorescein probe (DCF-DA) and nitroblue tetrazolium (NBT) solution. It also assessed the protective effect of the antioxidants (ascorbic acid, gallic acid and green tea Kombucha) to the cells upon treatment with mycolactone using cell viability assays. The mechanism of cell death induced by the mycolactone was investigated using fluorescence microscopy. The expression levels of endogenous antioxidant enzyme genes (catalase, superoxide dismutase and glutathione peroxidase) in response to mycolactone-mediated ROS were determined using RT-qPCR. Mycolactone induced the production of ROS in RAW 264.7 macrophages, whilst the ROS were scavenged by the antioxidants. The presence of some antioxidants enhanced the viability of the cells based on the model of treatment, hence the attenuation of the cytotoxic effects of mycolactone. The endogenous antioxidant enzymes were up-regulated in the presence of the antioxidants. The results provide insights into alternative therapeutic approaches in Buruli ulcer treatment, which could prevent or delay ulcer formation and possibly enhance wound healing.

DEDICATION

This project is dedicated to the Almighty God for the divine favour He bestows on me at every point in my life. To my husband, Jonathan Kwaffo, I render my profound gratitude for his endless support, care and love shown throughout the course of this programme. The reassurances and care from my mum, siblings and in-laws are very much cherished. Also, to Dr Lydia Mosi, I appreciate her support and belief in this work right from day one.

TABLE OF CONTENTS

DECLARATION	i
ABSTRACT.....	ii
DEDICATION.....	iii
TABLE OF CONTENTS.....	iv
ACKNOWLEDGEMENTS.....	viii
LIST OF FIGURES	ix
LIST OF TABLES.....	xi
LIST OF APPENDICES.....	xii
LIST OF ABBREVIATIONS.....	xiii
CHAPTER ONE.....	1
1.0 INTRODUCTION	1
1.1 Background.....	1
1.2 Rationale	3
1.3 Hypothesis.....	4
1.4 Aim	4
1.5 Specific objectives	5
CHAPTER TWO	6
2.0 LITERATURE REVIEW	6
2.1 BURULI ULCER DISEASE	6
2.1.1 History and Epidemiology	6
2.1.2 Clinical Features and Pathogenesis.....	7
2.1.3 Transmission.....	9
2.1.4 Treatment and Control Strategies of Buruli Ulcer.....	10
2.1.5 Diagnosis.....	11
2.1.6 Disease Management	12
2.1.7 Mycolactone, the Macrolide Toxin.....	13
2.1.7.1 Structure of mycolactone	14
2.1.7.3 Molecular targets of mycolactone.....	16
2.2 REACTIVE OXYGEN SPECIES	19
2.3 ANTIOXIDANTS.....	20
2.3.1 Endogenous Antioxidants	22

2.3.1.1	Glutathione peroxidase	22
2.3.1.2	Manganese superoxide dismutase.....	22
2.3.1.3	Catalase	23
2.3.2	Exogenous Antioxidants	24
2.3.2.1	Phenolic compounds	25
2.3.2.1.1	Gallic acid	25
2.3.2.2	Kombucha tea	26
2.4	TRANSCRIPTIONAL REGULATION OF ENDOGENOUS ANTIOXIDANTS	27
2.5	ACTIVITIES OF EXPRESSED ENDOGENOUS ANTIOXIDANT ENZYMES	28
2.6	WOUND HEALING	29
2.6.1	Role of Macrophages in Wound Healing.....	30
2.6.2	Effect of Excessive ROS on Wound Healing	31
CHAPTER THREE	32
3.0	MATERIALS AND METHODS.....	32
3.1	MATERIALS.....	32
3.2	METHODS	32
3.2.1	Preparation of Mycolactone Working Concentrations and Antioxidants (Ascorbic acid, Gallic acid and Green tea Kombucha)	32
3.2.1.1	Preparation of mycolactone working concentrations.....	32
3.2.1.2	Ascorbic and gallic acid preparation.....	32
3.2.1.3	Preparation of Kombucha tea.....	32
3.2.2	Determination of Antioxidant Activity of Black and Green Tea Kombucha	33
3.2.2.1	DPPH radical quenching activity.....	33
3.2.2.2	Total phenolic content of BTK and GTK	33
3.2.2.3	Total flavonoid content of BTK and GTK.....	34
3.2.3	Cell Culture.....	34
3.2.4	Cytotoxicity and Cytopathic Assay	34
3.2.4.1	Cytotoxicity of mycolactone and antioxidant compounds against Raw 264.7 macrophages	34
3.2.4.2	Cell viability using Alamar blue assay	35
3.2.5	Measurement of Reactive Oxygen Species (ROS)	35
3.2.6	Measurement of Superoxide Anions.....	36
3.2.7	Determination of Antioxidants Effect on Induced Intracellular Superoxide Anions and Cytoprotection.....	36
3.2.8	Measurement of Apoptosis and Necrosis	38
3.2.9	Gene Expression Analysis	39

3.2.9.1 Total RNA extraction.....	39
3.2.9.2 Quantitative reverse transcription PCR assay (RT-qPCR)	39
3.2.9.3 Primer set for PCR	40
3.2.10 Statistical Analysis.....	40
CHAPTER FOUR.....	41
4.0 RESULTS	41
4.1 DPPH SCAVENGING ACTIVITY OF BLACK AND GREEN TEA KOMBUCHA.	41
4.2 TOTAL PHENOLIC CONTENT OF BLACK AND GREEN TEA KOMBUCHA	42
4.3 TOTAL FLAVONOID CONTENT OF BLACK AND GREEN TEA KOMBUCHA.	43
4.4 MYCOLACTONE IS CYTOTOXIC TO RAW 264.7 MACROPHAGES	44
4.5 CYTOTOXICITY OF ANTIOXIDANTS ON RAW 264.7 CELLS	46
4.6 MYCOLACTONE INDUCES ROS PRODUCTION IN RAW 264.7 MACROPHAGES	47
4.7 PRE-/CO- AND POST-TREATMENT WITH ANTIOXIDANTS AND MYCOLACTONE.....	49
4.8 ANTIOXIDANT PROTECTION AGAINST MYCOLACTONE CYTOTOXICITY	51
4.8 (A) Cell Viability of RAW 264.7 Macrophages in Antioxidant Pre-treatment Model	51
4.8 (B) Cell Viability of RAW 264.7 Macrophages in Co-/Post-Treatment Model.....	53
4.9 MECHANISM OF CELL-DEATH IN MYCOLACTONE-TREATED RAW 264.7 MACROPHAGES	54
4.10 EFFECT OF ANTIOXIDANTS ON THE EXPRESSION LEVELS OF ENDOGENOUS ANTIOXIDANT GENES.....	56
4.10.1 Antioxidants Enhance Activity of Endogenous Antioxidant Genes in the Pre- treatment Model.....	56
4.10.2 Antioxidants Enhance Activity of Endogenous Antioxidant Genes in the Co- treatment Model	57
4.10.3 Antioxidants overly Enhance Activity of Endogenous Antioxidant Genes in the Post-treatment Model.....	58
CHAPTER FIVE	60
5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS	60
5.1 DISCUSSION	60
5.2 CONCLUSION.....	68
5.3 RECOMMENDATIONS	69
5.4 LIMITATIONS	69
6.0 REFERENCES	70

7.0 APPENDIX.....93

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LIST OF FIGURES

Fig 2.1: The stages of Buruli ulcer disease.....	8
Fig 2.2: Clinical presentations of BU.....	8
Fig 2.3: Complications of BU.....	8
Fig 2.4: Postulated sources of <i>M. ulcerans</i> infection.....	9
Fig 2.5: Chemical structure of mycolactone A/B.....	15
Fig 2.6: Molecular targets of mycolactone.....	17
Fig 2.7: Activation of Protein C by thrombin-thrombomodulin complex.....	18
Fig 2.8: Classification of endogenous and non-endogenous antioxidants	21
Fig 2.9: ROS-generating and -scavenging enzymes.	24
Fig 2.10: Chemical classification of phenolic compounds.	25
Fig 2.11: The phases of wound repair processes.	30
Fig 4.1A: Antioxidant activity of green and black tea Kombucha.....	41
Fig 4.1B: Antioxidant activity of BHT.....	42
Fig 4.2: Total phenolic content of black and green Kombucha tea.....	43
Fig 4.3: Total phenolic content of black and green Kombucha tea.....	44
Fig 4.4A: Cytotoxicity of mycolactone on RAW 264.7 macrophages.....	45
Fig 4.4B: Concentration-response curve for mycolactone-induced RAW 264.7 macrophage.....	45
Fig 4.5: Cytopathic effects of mycolactone on RAW 264.7 macrophages.....	46

Fig 4.6A: ROS levels in mycolactone-treated RAW 264.7 macrophages.....	48
Fig4.6B: Levels of intracellular superoxide anions in mycolactone-treated RAW 264.7 macrophages.....	49
Fig 4.7: Effect of antioxidants on superoxide anion production in mycolactone-treated RAW 264.7 macrophage.....	50
Fig 4.8: Cell viability of pre-treated RAW 264.7 macrophages with antioxidants and mycolactone.....	52
Fig 4.9: Cell viability of co and post-treated RAW 264.7 macrophages with antioxidants and mycolactone.....	53
Fig 4.10: Time-dependent protection of RAW 264.7 macrophages in the presence of antioxidants and mycolactone.....	54
Fig 4.11A: Photographs showing apoptotic and necrotic cell death.....	55
Fig 4.11B: Percentage of average number of apoptotic and necrotic cells.....	55
Fig 4.12: Gene expression of <i>SOD2</i> , <i>CAT</i> , <i>GPX1</i> in antioxidants pre-treated and mycolactone post-treated cells.....	57
Fig 4.13: Relative gene expression of <i>SOD2</i> , <i>CAT</i> , <i>GPX1</i> in antioxidant and mycolactone co-treated cells.....	58
Fig 4.14: Relative gene expression of <i>SOD2</i> , <i>CAT</i> , <i>GPX1</i> in mycolactone pre-treated and antioxidants post-treated cells.....	59

LIST OF TABLES

Table 3.1: List of primers used for PCR amplifications.....	40
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LIST OF APPENDICES

Fig 1: Calibration curve of gallic acid.....	93
Fig 2: Calibration curve of quercetin.....	93
Fig 3: Cytotoxicity of ascorbic acid on RAW 264.7 macrophages.....	94
Fig 4: Cytotoxicity of green Kombucha tea on RAW 264.7 macrophages.....	94
Fig 5: Cytotoxicity of gallic acid on RAW 264.7 macrophages.....	95

LIST OF ABBREVIATIONS

7-AAD	7-aminoactinomycin D
AFB	Acid fast bacilli
AP-1	Activating Protein 1
AP-2	Activating Protein 2
AREs	Antioxidant response elements
AT2R	Angiotensin 2 receptor
BHT	Butylated hydroxytoluene
BTK	Black tea Kombucha
BU	Buruli ulcer
C/EBP	CCAAT-Enhancer-Binding Proteins
CAT	Catalase
DAPI	4',6-diamidino-2-phenylindole
DCFDA	2',7'-dichlorofluorescein diacetate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EC	Effective concentration
EDTA	Ethylenediaminetetraacetic acid
Erk	Extracellular signal-regulated kinases

FAK	Focal adhesion kinases
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GA	Gallic acid
GAE	Gallic acid equivalent
GPX	Glutathione peroxidase
GSH	Monomeric glutathione/ reduced glutathione
GTK	Green tea Kombucha
JNK	c-Jun N-terminal kinases
KT	Kombucha tea
ML	Mycolactone
Mn-SOD	Manganese superoxide dismutase
MU	<i>Mycobacterium ulcerans</i>
NAC	N-acetyl-L-cysteine
NADPH	Nicotinamide adenosine dinucleotide phosphate
NBT	Nitroblue tetrazolium
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
PCR	Polymerase chain reaction
QE	Quercetin equivalent
ROS	Reactive oxygen species

RT-qPCR	Reverse Transcriptase Quantitative PCR
SCOPY	Symbiotic colony of bacteria and yeast
SOD	Superoxide dismutase
Sp1	Specificity Protein 1
TBHP	Tert-butyl hydroperoxide
TRITC	Tetramethylrhodamine
WASP	Wiskott Aldrich syndrome protein
ZN	Ziehl-Neelsen

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Buruli ulcer (BU) is a skin disease characterized with necrosis, with *Mycobacterium ulcerans* (MU) as its causative agent. The disease was first described in Australia as Bairnsdale ulcer (MacCallum *et al.*, 1948) and subsequently called Buruli ulcer in Uganda (Clancey *et al.*, 1961). It starts as nodule or plaque but progresses when there is delayed detection, diagnosis and treatment (Wansbrough-Jones and Phillips, 2006). The disease characteristically affects the extremities mostly than on the trunk. Although its route of transmission is not known and considered a non-contagious disease, insects living in water bodies have been implicated in several studies (Marston *et al.*, 2002).

M. ulcerans secretes a macrolide toxin called mycolactone (ML) which is cytotoxic, and the main virulence factor in the pathogenesis of BU due to its ability to prime cells for apoptosis and necrosis leading to ulcers. This property has been shown on most cell types and modulates the activity of immune cells (George *et al.*, 2000); however, the mechanism by which mycolactone exerts its toxicity has remained obscure until recently. It is also reported that, some macrolide substances with cytotoxic and immunosuppressive properties induce the production of reactive oxygen species [ROS] (Navarro-Antolín *et al.*, 1998). In a study conducted by Grönberg *et al.* (2010), *M. ulcerans*' mycolactone induced the production of ROS in keratinocytes, as treatment with antioxidants exerted protective effect on the keratinocytes which are critically involved in wound repair, by forming an epithelial barrier that protects the wound bed. Due to the disruption of the well-ordered process of wound healing by mycolactone, the expected time frame for the wound closure is delayed resulting in the development of chronic wounds which raises public health concern (Harding *et al.*, 2002).

Reactive oxygen species (ROS) are chemical species that have one or more unpaired electrons in their outer orbit making them unstable and highly reactive (Tiwari *et al.*, 2017). To attain a stable state, these free radicals interact with molecules with which they have high affinity for, thereby abstracting hydrogen atom from them. Some of such molecules are lipids, proteins, carbohydrates and nucleic acids thus damaging them structurally and functionally (Sivanandham, 2011). Intracellularly generated ROS are mainly oxygen radicals such as superoxide, hydrogen peroxide and the hydroxyl radical (Kunwar and Priyadarsini, 2011). Establishment of an equilibrium between ROS generation and antioxidant defense system in an organism is critical for its normal physiology. However, under pathological conditions where ROS is produced excessively, a shift in balance of ROS and antioxidant defense mechanisms leads to cell membrane modification through lipid peroxidation.

Structural changes in intracellular molecules (Büyükokuroğlu *et al.*, 2001) such as nucleic acids and proteins also occur, therefore, the necessity to minimise or scavenge ROS activity within cells using natural antioxidants. Again, in response to cutaneous injury, ROS are produced (Gupta *et al.*, 2002) and may induce cellular damage by inactivating sulphhydryl enzymes, cross-linking proteins and breaking down DNA (Russo *et al.*, 2002). It is therefore suggestive that, healing outcome may be enhanced and beneficial when local antioxidants are used to counteract the effects of the ROS (James *et al.*, 2001). Increased ROS formation has been elucidated to result in apoptosis (Buttke and Sandstorm, 1995) while the addition of antioxidants has been proven to block or slow down apoptosis (van den Dobbelen *et al.*, 1996).

Ascorbic acid is a well-known water-soluble molecule that reduces transition metals as well as scavenge free radicals in biological systems (Kaur and Kapoor, 2001). It is mostly present in the cytosol also donates electrons to reactive species to stabilize them and neutralize their deleterious effects (Frei *et al.*, 1989). Vitamin C is also involved in the recycling of vitamin E

to restore their active reduced state. Several other reducing substances such as vitamins A and E) together with GSH, urate, ubiquinone are present in all cells to scavenge reactive molecules (Sauer *et al.*, 2001). Radical trapping antioxidants vitamin A and E (lipid soluble) reduce peroxy radicals to prevent generation of hydroperoxides which are involved in the peroxidation of membrane lipids (Palace *et al.*, 1999).

Gallic acid (3,4,5-trihydroxybenzoic acid, GA) is an antioxidant obtained from plants, especially green tea (Lu *et al.*, 2006) and has wide usage in foods, cosmetics and drugs. It has received much attention over the years due to its potent antioxidant activity of scavenging free radicals (Kim, 2007).

Kombucha tea (KT) is a fermented tea which has been indicated to exert medicinal properties through the co-existence of yeast and bacteria. A study by Jayabalan *et al.* (2014) indicated that, ascorbic acid and tea polyphenols were the contributing factors to the antioxidant activity of Kombucha tea. These properties make it a therapeutic agent for the treatment and prevention of diseases such as arthritis (Sreeramulu *et al.*, 2000) due to its antioxidant activities (Jayabalan *et al.*, 2014). In another research, Kombucha tea has been shown to reduce lipid peroxidation and oxidative stress (Gharib, 2009). Ofori *et al.* (2015) demonstrated that Kombucha tea protects rats against protein oxidation induced by arsenic, thus the use of Kombucha tea as an antioxidant in this study; a good choice.

1.2 Rationale

Mycolactone with its multi-functional cytotoxicity is known to stabilize and increase the survival rate of *Mycobacterium ulcerans* in host infected cells and aids their release into the extracellular space, thus spreading to infect other cells (Rondini *et al.*, 2006). High concentrations of ML locally cause massive tissue destruction leading to extensive ulcerations and death of surrounding cells, more importantly immune cells. Even at its lower

concentration it causes immunosuppression of white blood cells and hence inhibits the host's immunological response. According to a study by Sarfo *et al.* (2014), there is persistence of ML after the 8 weeks of treatment with antibiotics which is an indication of the continuous exposure of cells and tissues to the cytopathic effects of the toxin even after complete elimination of the mycobacterium.

ML has been associated with the stimulation of ROS production where generated ROS also impair wound healing processes, hence the delay in wound healing. To counteract the mycolactone mediated ROS production, the search for natural antioxidants which are presumed to be potent, safe and possessing high therapeutic value is necessary. Even though ascorbic acid, gallic acid and green tea Kombucha have been proven to exhibit potent antioxidant activities, there is no evidence of the role they may play in ameliorating the toxicity of mycolactone, which may eventually ensure protection of wound-healing cells such as macrophages, keratinocytes and fibroblasts. Despite the key role macrophages play in modulating fibroblast and keratinocyte numbers during wound healing, no studies have been reported on mycolactone-induced ROS activity in them, and the effect natural antioxidants could exert on them *in vitro*. Furthermore, the direct effect of mycolactone on the level of expression of antioxidant enzyme genes has not been reported.

1.3 Hypothesis

Antioxidants attenuate the cytotoxic effects of mycolactone by neutralizing ROS and enhancing endogenous antioxidant enzyme genes.

1.4 Aim

To determine the effect of mycolactone mediated ROS activity on RAW 264.7 macrophages in the presence of natural antioxidants.

1.5 Specific objectives

- To determine ROS activity in RAW 264.7 macrophages in the presence of ML.
- To determine the effect of antioxidants (ascorbic acid, gallic acid and green tea Kombucha) on ML-treated ROS and their protective effect on the cells.
- To identify level of expression of ROS responsive enzymes; (superoxide dismutase, catalase and glutathione peroxidase) in antioxidant-treated and ML-treated cells using quantitative reverse transcription PCR.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 BURULI ULCER DISEASE

2.1.1 History and Epidemiology

Buruli ulcer (BU) was previously reported and described as a chronic ulcer in The Mengo Hospital Notes in Kampala, Uganda in 1897 by a physician from Britain called Sir Albert Cook (Cook, 1897). In the 1940s and 1950s, about 170 patients living in Belgian Congo which is currently called the Democratic Republic of the Congo, experienced chronic ulcers caused by mycobacteria (Janssens *et al.*, 1959). The first ever publication was made by MacCallum and Tolhurst in 1948 where they isolated mycobacterium as an etiologic agent from six patients and later called it *M. ulcerans*. This report outlined various stages of Buruli ulcer disease in two children from Australia and four adults hailing from Bairnsdale in Victoria (MacCallum *et al.*, 1948). In the 1960s, there were reports of high number of cases in Uganda, specifically Buruli County, thus bringing forth the name, Buruli ulcer (Clancey *et al.*, 1961).

Decades down the line, *M. ulcerans* lineages that are pathogenic to humans have been reported in 34 countries which are predominantly in the tropics and sub-tropics. Although there have been reported incidences of sporadic BU cases in some regions where the *M. ulcerans* originates, Australia (Veitch *et al.*, 1997) and Africa have been shown to have the highest foci of the infection with high incidence rates present in several West African countries such as Benin (Debacker *et al.*, 2004), Côte d'Ivoire (Marston *et al.*, 1995), Ghana (Amofah *et al.*, 2002) and Cameroon (Noeske *et al.*, 2004). In 1998, World Health Organization (WHO) set up the Global Buruli Ulcer Initiative to publicize and organize global BU control and research efforts (Wansbrough-Jones and Phillips, 2006) due to under-

reported BU cases in endemic African countries, that was revealed from surveillance studies (Amofah *et al.*, 2002).

Currently, active report of BU cases stem from 13 countries in Africa (Cameroon, Côte d'Ivoire, Benin, Gabon, Togo, Ghana, Guinea, Democratic Republic of the Congo and Nigeria), the Western Pacific (Australia), Asia (Japan) and Americas [French Guiana] (WHO, 2015). In the last 10 years there has been worldwide reports of more than 42,000 BU cases in 20 countries, however, there has been decline in the number of actively reporting BU country cases as WHO in 2014 reported about 2,251 cases (WHO, 2015). About 50% of the affected individuals are children under 15 years in the rural areas who are incapable of accessing health facilities (Asiedu *et al.*, 2000) and thus present late for medical treatment leading to extensive lesions that results in deformities (Asiedu and Etuaful, 1998).

2.1.2 Clinical Features and Pathogenesis

Infection with *M. ulcerans* usually begins as a nodule which is normally curative (Van der werf *et al.*, 1999), but due to the absence of inflammatory response and painless appearance medical attention is not sought at the initial stages of the disease. According to the WHO, three classifications of BU exist based on diameter of the lesions namely; category I lesions (less than 5 cm), category II lesions (5–15 cm) and category III lesions [more than 15 cm as well as lesions at critical sites (breast, genitalia, eye,), osteomyelitis and multiple lesions] (WHO, 2012). Amidst socio-cultural beliefs and similarity of BU to other skin diseases, they are left undiagnosed and untreated which then progresses to the ulcerative stage within few weeks to months to form large necrotic ulcers of the dermis and subcutaneous adipose tissues usually characterized with undermined edges (Raghunathan *et al.*, 2005), but in complicated cases underlying bones become affected (Portaels *et al.*, 2001).

Because the rate of growth of MU is very slow, its exact period of incubation to disease development is not clear. The disease has low rate of mortality but high rate of morbidity as 66% of people have their lesions heal with disability (Ellen *et al.*, 2003) and affects people in the age brackets of 12 years.

Most patients present lesions occurring on the lower limbs, upper limbs, neck, head and the trunk. The pre-ulcerative and ulcerative stages of the disease (Fig 2.1), clinical presentations (Fig 2.2) and complications of BU (Fig 2.3) are shown below.

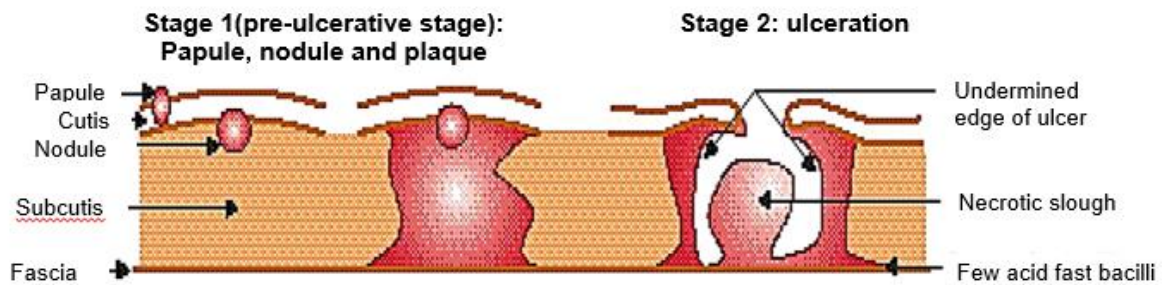


Fig 2.1: The stages of Buruli ulcer disease (Van der Werf *et al.*, 1999).



Fig 2.2: Clinical presentations of BU

Fig 2.3: Complications of BU

- A) Nodule B) Oedema C) Plaque
- D) Ulcer E) Osteomyelitis

(Capela *et al.*, 2015).

2.1.3 Transmission

Possoms have been indicated to be a reservoir of *M. ulcerans* in BU focus of southeastern part of Australia (Fyfe *et al.*, 2010) with mosquitoes being posited as the possible vectors (Johnson and Lavender, 2009). BU endemic regions of Africa have no evidence of animal reservoir or mosquito vectors and epidemiological studies conducted in Cameroon and Ghana suggested the unlikely involvement of mosquitoes in the transmission of *M. ulcerans* due to the early exposure of malaria parasites to children, causing malaria and yet no reportage of *M. ulcerans* infections (Röltgen *et al.*, 2014). However, humans with active BU disease showing ulcerative lesions could be possible dissemination point of *M. ulcerans* in endemic areas of Africa (Röltgen and Pluschke, 2015).

Contact with *M. ulcerans*-contaminated water (Fig 2.4) has been indicated in recent studies as possible source of transmission since decaying organic matter under the water can harbor these organisms for many months (Bratschi *et al.*, 2014). Other studies have also reported that *M. ulcerans* accumulated in water-filtering organisms (present in plant biofilms, mud or detritus) can be passed on to predatory animals that feed on these water-filtering organisms (Merritt *et al.*, 2010). Wounds, abrasions or lacerations that get exposed to environmental reservoirs of *M. ulcerans* may also become infected (Portaels *et al.*, 2008).



Fig 2.4: Postulated sources of *M. ulcerans* infection

2.1.4 Treatment and Control Strategies of Buruli Ulcer

Until 2004 when antibiotic therapy was introduced, surgery remained the major and effective treatment of BU disease which involved the removal of all infected tissues as well as healthy tissues. Multiple operations are associated with this method of treatment during excision and skin grafting requiring an average of 3 months hospitalization (Asiedu and Etuaful, 1998). This prolonged hospitalization puts lots of constraints on the limited number resources for surgical procedures as well as bed capacity of health centers in these rural endemic regions, hence reducing the number of treated patients. The high cost of surgery is also unaffordable by these impoverished patients (Grietens *et al.*, 2008) and many have little confidence in the surgical interventions due to the recurrence rate ranging from 16% to 28% (Kibadi *et al.*, 2009), which usually occurs without post-operative chemotherapy thus inflating costs of treatment.

The introduction of antibiotic treatment has drastically reduced the recurrence rate to 0–2% thus minimizing the need for surgical interventions (Sarfo *et al.*, 2010). There exists enough evidence of the effectiveness of antibiotic therapy, specifically rifampicin and streptomycin co-administration in the complete healing of small early lesions during the 8 weeks of treatment (WHO, 2012). This has been demonstrated in observational studies in both Ghana (Sarfo *et al.*, 2010) and Benin (Chauty *et al.*, 2007). In another study, the efficacy of antibiotics was proven during histological analysis of tissues prior and post treatment (Schütte, *et al.*, 2007) with the most aggressive form of this disease, edematous lesions being responsive to the antibiotic treatment. A comparative study in Ghana sought to investigate whether oral administration of clarithromycin could be a good substitute of intramuscular injection of streptomycin and was proven to have no significant difference in their outcome (Phillips *et al.*, 2014), however, it has not been tested in clinical trials although it has been

replicated and clinically assessed in Australia (O'Brien *et al.*, 2012) as well as French Guiana (WHO, 2012) which gives an indication of the effectiveness of oral regimens.

2.1.5 Diagnosis

There are currently four methods for diagnosis of BU in the laboratory and the most readily available method used in the rural treatment centers is the microscopy, for direct smear examination of wound exudates; to detect acid fast bacilli (AFB) using Ziehl-Neelsen/Methylene blue staining. Irrespective of its easy application and quick results delivery, microscopy is associated with low sensitivity, shows no differentiation between viable and non-viable bacilli and cannot also differentiate BU from cutaneous tuberculosis (Portaels *et al.*, 2001). The remaining three methods are mostly performed in the tertiary or referral hospitals due to their requirement of sophisticated equipment and need for highly trained professionals. Cultivation of *M. ulcerans in vitro* allows the differentiation of viable and non-viable organisms and helps in treatment and drug resistance monitoring. However, due to the slow growth rate of *M. ulcerans* and its low sensitivity, this method is usually used for immediate patient care (Portaels *et al.*, 2001).

Histopathology involves sampling procedures that are invasive due to the sampled biopsies. This technique is helpful in monitoring treatment response and rate of wound healing thus providing insights into the pathogenesis of the disease (Andreoli *et al.*, 2014). The gold standard for BU diagnosis currently is polymerase chain reaction (PCR) that targets an insertional sequence 2404 (IS2404) and regarded as the most specific and sensitive in comparison to the other three methods (Portaels *et al.*, 2001). However, its use requires regular and stringent quality control measures to obtain accurate PCR results that excludes either false positive or negative results (Eddyani *et al.*, 2014). Current recommendations from WHO necessitate health professionals to use at least one of the four methods for the confirmation of suspected BU cases and most preferably by obtaining positive results from at

least two different tests to minimize misdiagnosis. It is again recommended that at least 70% of reported BU cases get confirmed by PCR (WHO, 2015).

During laboratory confirmation of BU, different sampling methods may be used which are dependent on the nature of the lesion (open or closed). For open ulcerative lesions cotton swab are used for swabbing whereas early closed lesions require fine-needle aspiration (Yeboah-Manu *et al.*, 2011). Any of these sample types, be it closed or open lesion can be examined in the laboratory via microscopy, culturing and PCR. Point-of-care diagnostic tests, mycolactone detection in human lesions as well as *M. ulcerans*-specific antibodies diagnostic testing could be possible approaches in the easy diagnosis of BU (WHO, 2015).

2.1.6 Disease Management

All clinical presentations of Buruli ulcer are responsive to antibiotic chemotherapy irrespective of its extensiveness but large lesions require surgery due to their slow healing rate. Antibiotic treatment periods may be characterized with paradoxical reactions usually between 2–12 weeks post antibiotic therapy whilst some may present over a year later (Ruf *et al.*, 2011). Due to this immunological response lesions may deteriorate faster after initiation of chemotherapy, develop at other parts of the body or even ulceration of previously non-ulcerative forms can occur. However, they can be resolved without any form of treatment other than the completion of the 8-week chemotherapy amidst regular wound dressing and physiotherapy to minimize scar formation. Scarring and adhesions are serious complications which can restrict movement if found near joints resulting in long-term disability (Simonet, 2008).

2.1.7 Mycolactone, the Macrolide Toxin

Organisms belonging to the order Actinomycetales are well known producers of secondary metabolites such as macrolides; which are lipid-like in nature with potent biological activity. Erythromycin (antibiotic), avermectin (antihelmetic) and amphotericin B (antifungal) are common examples of macrolides (George *et al.*, 1999). Since *M. ulcerans* belonged to this order, it was suspected to produce the macrolide too, as lot of observations were made by clinicians about the extent to which tissues got damaged without the presence of the *M. ulcerans* in that foci. This led to the hypothesis that there was a possible exotoxin produced by the bacterium that diffuses in the dermis to expand the ulcers (Connor and Lunn, 1965). Multiple efforts in identifying and characterizing the mycolactone toxin proved futile when preliminary data reported it to be a proteinaceous and heat stable substance present in sterile culture filtrate (Hockmeyer Jayabalan *et al.*, 1978).

In 1974, it was postulated by Kreig and colleagues to be a polysaccharide complex composed of phosphoprotein, where they subjected broth cultures of *M. ulcerans* to protease enzymes to digest the suspected proteins (Kreig *et al.*, 1974). It was, however, observed that the cultured broth continued to exert its cytopathic effects on L929 fibroblast cell cultures even after the protease treatment, thus suggested that it was not proteinaceous. Alternatively, the broth culture was subjected to heat with the aim of inactivating it to prevent its cytotoxic effect, yet again it proved to be insensitive to heat thus non- protein (George *et al.*, 1998). Investigating further on the study, exponential phase of *M. ulcerans* cultures were extracted and subjected to thin layer chromatography to obtain separate bands of lipids which were then tested to be toxic to L929 murine fibroblasts in cell culture thus confirming earlier suspicions of the exotoxin being lipid (George *et al.*, 1998). This toxin was later described in 1999 as a lipid-like polyketide earning the name Mycolactone (George *et al.*, 1998).

2.1.7.1 Structure of mycolactone

Mycolactone is a polyketide-derived 12 membered macrolide ring which is ester-linked to a polyketide side chain (George *et al.*, 1999) (Fig 2.5). Its synthesis depends on the presence of 174 kb virulence plasmid pMUM, found in all mycolactone-producing mycobacteria (MPM) and the polyketide synthases required for the synthesis of ML is encoded in this plasmid (Stinear *et al.*, 2004).

Currently, about six isoforms of the ML have been identified with different geographical distribution namely: A to F with each of these variants containing the same lactone core but different lower side chains varying in length, number and positions of double bonds and hydroxyl groups, thus resulting to the different potency and cytotoxicity (Kishi, 2011). This makes the lower side chain the determining factor in the biological activity of ML. The most potent isoform is the mycolactone A/B that is produced by Africa's most virulent *M. ulcerans* strains, with its lower side chain having a 3:2 ratio of Z-/E- isomers of the C-4-C-5 bond. The predominant *M. ulcerans* strains in Asia and Australia produce mycolactone C and D respectively which are less potent in comparison to the African strain (Mve-Obiang *et al.*, 2003). Apart from the ML A/B, C and D which are human pathogenic strains of *M. ulcerans*, there is existence of fish and frog pathogenic strains that were previously named as *M. pseudoshottsii* and *M. liflandii*, respectively, which produce the mycolactone E and F (Hong *et al.*, 2008).

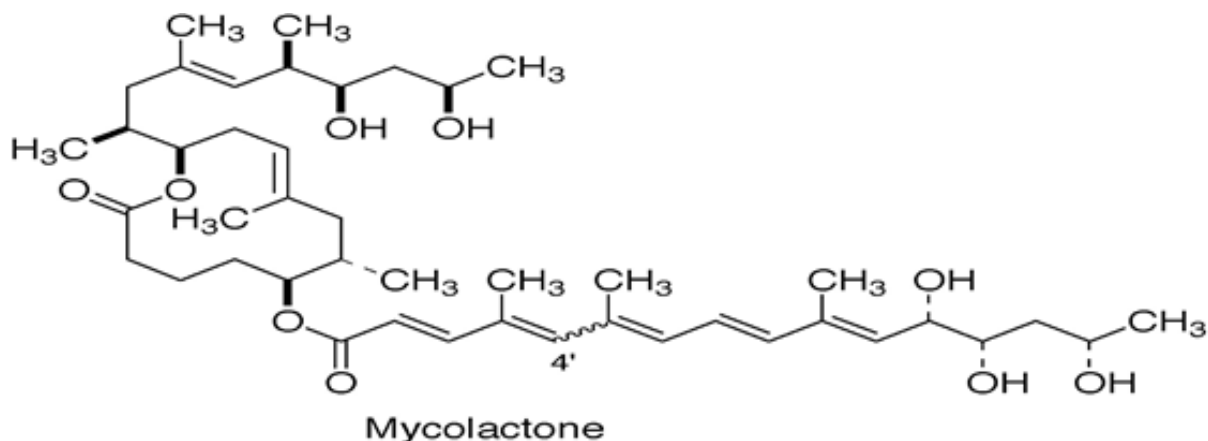


Fig 2.5: Chemical structure of mycolactone A/B (Ranger *et al.*, 2006).

2.1.7.2 Biological activity and cellular consequences of mycolactone

The two well-established functional characteristics of mycolactone are its cytotoxicity (with obscured underlying molecular mechanism) and immunosuppression (abundant evidence). It has been proven to modulate both local and systemic immune responses (Adusumilli *et al.*, 2005) for instance, the blockade of different immune cell activation such as macrophages, monocytes, T-cells and dendritic cells (Simmonds *et al.*, 2009). Due to its lipid-like nature (hydrophobic molecule), its entry into the cell is believed to be non-receptor mediated but rather a passive diffusion along the membrane lipids of the cell to localize in the cytoplasm and this was demonstrated in an experiment by Snyder and Small (2003), where mycolactone had been labelled with a fluorescein and was shown to diffuse passively across cell membranes of exposed fibroblast. However, in another study there was observation of the localization of Bodipy-mycolactone in the nucleus of the cells (Chany *et al.*, 2011).

To ascertain the biological function of mycolactone A/B, many researchers have tried both *in vivo* and *in vitro* experiments and sought to make comparison between the effects of wild type MU and that of mycolactone- negative mutant strains. Injection of purified mycolactone alone could replicate Buruli ulcer disease characteristics such as redness, oedema and ulcers (George *et al.*, 1999), with similar observations made when bacterial cells were injected into

the dermis of guinea pigs. However, mycolactone-negative mutant strains could not replicate same effects (Adusumilli *et al.*, 2005). Culture filtrate of *M. ulcerans* has also been shown to be cytotoxic to L929 murine fibroblasts (Read *et al.*, 1974). Mycolactone as well, has been shown to have irreversible effect on the production of cytokines (Hall *et al.*, 2014) where there was evidence of cytokine suppression even after 24 to 48 hours of removal of mycolactone from the cell culture media. Concentrations of mycolactone between (15 ng/mL to 150 ng/mL) or above 15 µg/mL have been reported to induce apoptosis and necrosis, respectively, in L929 fibroblast after 4-24 hours of exposure (Adusumilli *et al.*, 2005). While a few number of cells exert resistance to mycolactone (Bozzo *et al.*, 2010), several cells have remained susceptible to the cytopathic effects of mycolactone some of which are adipocytes (Dobos *et al.*, 2001), monocytes (George *et al.*, 2000), epithelial cells (Bozzo *et al.*, 2010; Gronberg *et al.*, 2010).

Fibroblasts (George *et al.*, 1999) as well as keratinocytes (Gronberg *et al.*, 2010) have also been demonstrated to be killed at lower concentrations of the ML. Mycolactone-induced cell death in keratinocytes has been linked to the over-production of reactive oxygen species (ROS) in a study by Gronberg and colleagues (2010) which inferred that the use of antioxidants could be an inhibitor of the ROS thus preventing cell death. In their study, iron chelating substance deferoxamine was able to inhibit cell death giving an indication of the involvement of Fe²⁺-dependent hydroxyl radicals whose production might have been interrupted due to the iron chelating properties of deferoxamine.

2.1.7.3 Molecular targets of mycolactone

Mycolactone is reported to alter the cytoskeletal rearrangement of cells through the direct binding and hyperactivation of Wiscott-Aldrich protein (WASP), thus leads to defective cell migration and adhesion [Fig 2.6A] (Guenin-Mace *et al.*, 2013) with subsequent arrest of the cell cycle at the G₀/G₁ phase which eventually induces apoptosis (George *et al.*, 2000).

Although ML has been demonstrated to inhibit production of several proteins such as the cytokines and chemokines, information on its direct effect on transcription or translation is scanty, but has rather been shown to alter the structure and function of a translocon, Sec61 [Fig 2.6B] (McKenna *et al.*, 2016) which is involved in the translocation of proteins to the endoplasmic reticulum thus getting locked up in the cytoplasm for rapid proteosomal degradation (Hall *et al.*, 2014). The exact mode of mechanism of the analgesic property of ML has not been well studied, however its involvement in nerve damage was proven in mice models when ML was injected into their footpads (En *et al.*, 2008), thus, explained the painlessness at the site of *M. ulcerans* infection.

Another study provided evidence that tended to contradict the hypothesis of ML-treated nerve damage since nerve destruction is observed in the late stages of the infection whereas painlessness is experienced right from the early stages of the infection (En *et al.*, 2008). That study showed that ML rather exerts its analgesic property via signal pathway using angiotensin II receptors (AT2Rs), thus leading to hyperpolarization of neurons (Marion *et al.*, 2014).

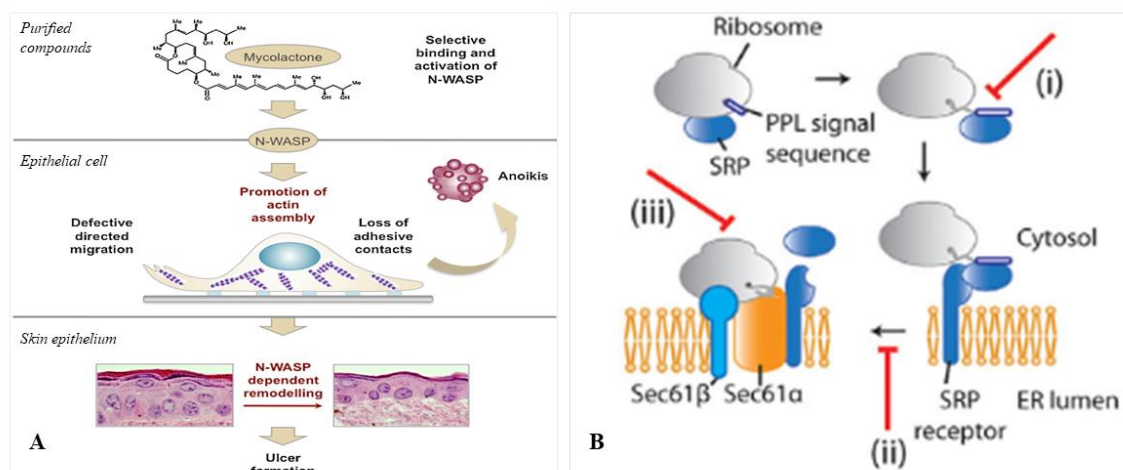


Fig 2.6: Molecular targets of mycolactone (A) Hyperactivation of neuronal Wiskott Aldrich syndrome protein (N-WASP) by mycolactone [Guenin-Macé *et al.*, 2013]. (B) Diagram showing the co-translational pathway indicating the possible sites of inhibition by mycolactone [(i), (ii) and (iii)] (McKenna *et al.*, 2016).

Just recently, a study by Ogbechi *et al.* (2015), reported of the sensitivity of endothelial cells' thrombomodulin to mycolactone in both in vitro and in vivo studies. This was suggested to lead to loss of coagulation control and eventual fibrin deposition in untreated BU lesions. Mycolactone was shown to deplete thrombomodulin of the endothelial cells, which then disrupts the protein C anticoagulant pathway (Fig 2.7). The possible mechanism that could lead to the thrombomodulin depletion were proposed by these researchers, which was the internalization of thrombin-thrombomodulin complex via endocytosis (Maruyama and Majerus, 1985) without the recycling of the thrombomodulin (TM) to the cell surface, due to their likely subjection to proteosomal degradation (Hall *et al.*, 2014). Their findings ultimately suggested that, the presence of mycolactone-orchestrated loss of TM leads to coagulation pathway disruption, resulting to tissue ischemia, hence the cytopathic effects seen.

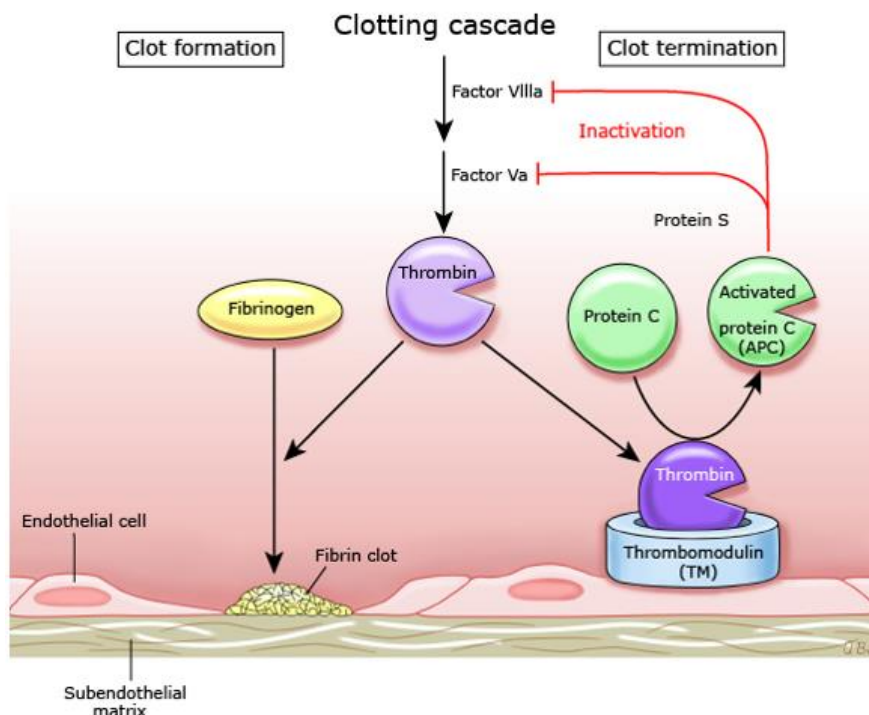


Fig 2.7: Activation of Protein C by thrombin-thrombomodulin complex

2.2 REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) are highly reactive molecules whose generation occurs due to reduction of their oxygen by electrons resulting in their high reactivity. Hydrogen peroxide (H_2O_2), peroxides (O_2^{-2}), superoxide anion ($\cdot\text{O}_2^-$) and hydroxyl radicals ($\cdot\text{OH}$) are some of the well-known molecules belonging to the ROS family (Bhattacharya, 2015). Primarily, they are majorly produced during mitochondrial electron transport chain, although peroxisomes, cytochrome P-450 metabolism, inflammatory cell activation and NADPH oxidases are also key endogenous sources of ROS (Finkel and Holbrook, 2000). Exogenously, ROS can be generated by use of X-rays, gamma rays, during UV light irradiation and metal-catalyzed reactions. They can also be produced during inflammatory response by macrophages, neutrophils as well as eosinophils (Shinde *et al.*, 2012).

Physiological functions of ROS include phagocytosis, cell signaling and homeostasis. With respect to cellular homeostasis, much evidence is available to suggest the active role of ROS in the maintenance of normal cell function as well as homeostasis at normal levels. At aberrant lower concentrations of ROS, cell cycle arrest can be induced. It has also been demonstrated that moderately high levels of ROS are generated for host defense against foreign pathogens (Bylund *et al.*, 2014).

If ROS are overly produced, they become accumulated in higher concentrations and ultimately overpowers the antioxidant defense system of the body (Devasagayam *et al.*, 2004), inducing oxidative stress. This leads to oxidative modification of key biological molecules such as nucleic acids, lipids and proteins (Moldovan and Moldovan, 2004). Oxidation of proteins involved in signal transduction alters downstream processes of the pathway hence, interrupting the expression of genes, growth and survival of cells (Allen & Tresini 2000). Eventually, these ROS can trigger the activation of pro-apoptotic markers directly or indirectly, therefore, inducing cell death by mechanisms such as apoptosis and

necrosis (Shen and Pervaiz, 2009). Furthermore, ROS has been reported to induce apoptosis by oxidizing cell membrane lipids, resulting in pore formation and disruption of mitochondrial membrane potential, which eventually contributes to the release of cytochrome C and caspase activation (Zamzami *et al.*, 1995). Reactive oxygen species (ROS) are therefore regarded generally as a double-edged sword with respect to their physiological and pathological roles in organisms (Day, 2014). There are, therefore, numerous studies that have demonstrated this by inhibiting apoptosis with anti-oxidative enzymes (Simon *et al.*, 2000).

2.3 ANTIOXIDANTS

Antioxidants inhibit or reduce the negative effects of ROS by donating their free electrons to the highly unstable reactive species, thereby stabilizing them to inhibit their abstraction of electrons from key biological molecules such as nucleic acids, lipids and proteins (Soares *et al.*, 2005). Antioxidants can be categorized as endogenous or exogenous depending on its source in nature. Endogenous antioxidants are further categorized into either non-enzymatic or enzymatic antioxidants. Examples of enzymatic antioxidants are superoxide dismutase (SOD), glutathione peroxidases (GPX), catalase (CAT), Thioredoxin 1 and 2, NADP (H) quinone oxidoreductase, heme oxygenase 1, glucuronosyl transferases (Mates, 2000) and key amongst these antioxidant enzymes are the manganese superoxide dismutase (MnSOD), glutathione peroxidase and catalase (Fig 2.8), which offer protection to cells from ROS produced by the mitochondria and peroxisomes (Moldovan and Moldovan, 2004). Non-enzymatic antioxidants can also be grouped into either nutrient antioxidant or metabolic antioxidant. Some of the nutrient antioxidants can be exogenous [natural foods such as vitamin E, carotenoids, phenolic compounds, vitamin C] (Willcox *et al.*, 2004) and trace elements (Mn, Zn, Cu); which have been shown to have high free radical scavenging activity (Kurahashi and Fujii, 2015). Examples of metabolic antioxidants are bilirubin, uric acid, glutathione, L-arginine, lipoic acid [Fig 2.8] (Kohen and Nyska, 2002).

Antioxidants have been proposed to work in the body in three different ways; firstly, by preventing excessive production of reactive species to keep them at minimal levels, with deferoxamine as example. Secondly, they can intercept or scavenge the reactive species using catalytic and non-catalytic molecules with examples being vitamin E and vitamin C. Thirdly, antioxidants are involved in the repair of molecules that have been damaged by the reactive species with glutathione playing major role (Zhivotovsky and Orrenius, 2011).

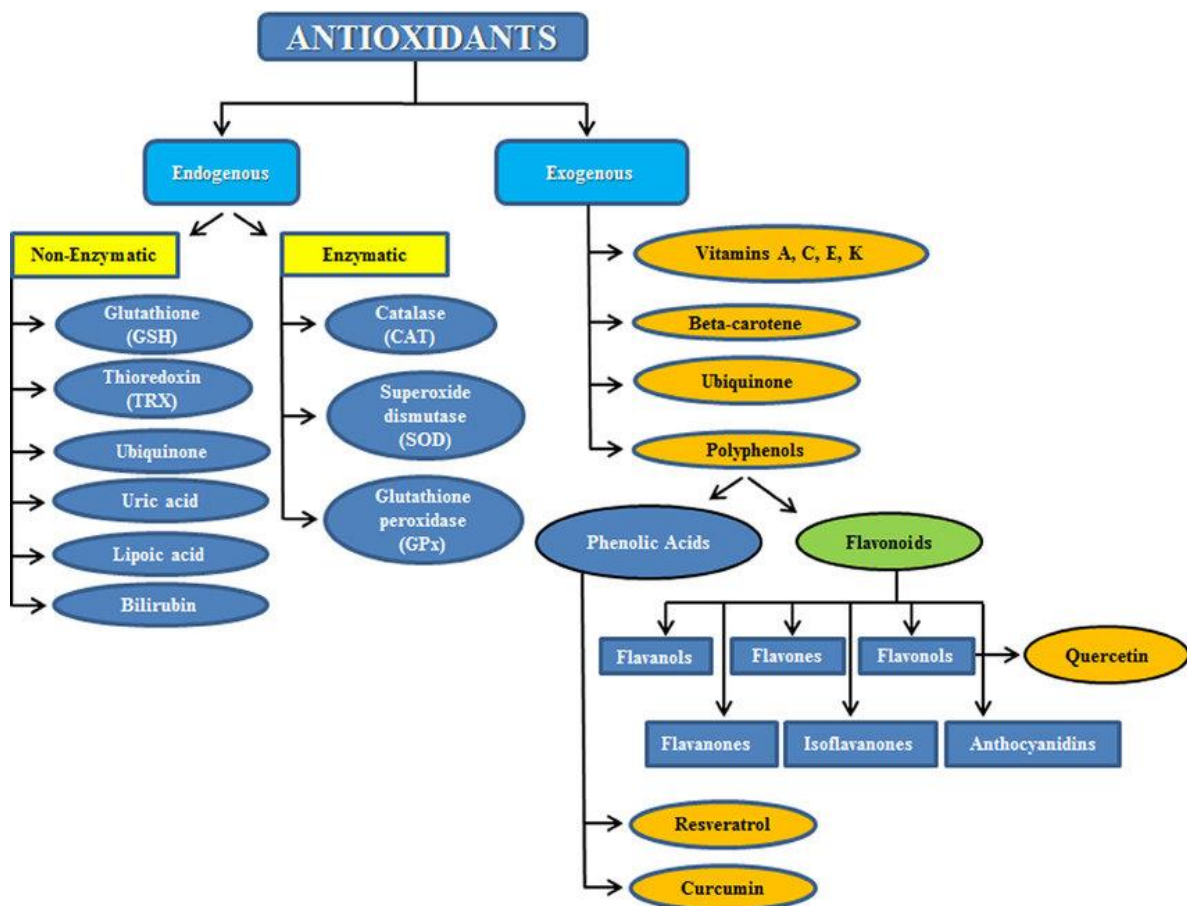


Fig 2.8: Classification of endogenous and non-endogenous antioxidants (Simioni *et al.*, 2018).

2.3.1 Endogenous Antioxidants

2.3.1.1 Glutathione peroxidase

Glutathione peroxidase (GPX) is an antioxidant enzyme whose active site contains selenium ion that efficiently reduces hydrogen peroxide to water. It also catalysis the conversion of lipid peroxides to lipid alcohols to prevent lipid peroxidation. Glutathione peroxidase, therefore, plays a key role in the preservation of cellular and subcellular membrane integrity. Several isoforms of glutathione peroxidase exist ranging from GPX1-GPX8 (1-4 identified and characterized) and differ based on their specificity to substrates and cellular localizations. GPX1 is the most abundant in the cytosol and mitochondria (Schafer and Buettner, 2001) and reduces lipid peroxides. The attached selenium at its active site also augments its function as a defense antioxidant by removing hydrogen peroxide using reduced glutathione (GSH). The GSH acts as a substrate to transfer electrons to the H_2O_2 forming two molecules of water (Fig 2.9).

Again, GSH can act as cofactor for glutathione transferase to remove reactive molecules, chemicals and drugs from cells (Kunwar and Priyadarsini, 2011). Intracellular glutathione levels have been shown to prevent Fas receptor-mediated apoptosis in cells (Watson *et al.*, 1997) with similar observation in eosinophils. Low activity of GPX and levels of glutathione have been characterized with hydrogen peroxide and lipid peroxide accumulation in cells due to their non-detoxification, thus leading to the generation of reactive hydroxyl and peroxy radicals by transition metals (Fe^{2+}).

2.3.1.2 Manganese superoxide dismutase

Superoxide dismutase (SOD) is of three types based on their location and their requirement of metal ions as co- factors to augment their function which are; Cu/Zn-SOD (SOD1), present in the cytosol, Mn-SOD (SOD2) also in the mitochondria and extracellular SOD (SOD3) which is found in the extracellular space. These SODs cause the dismutation of generated

superoxide anions to H_2O_2 and water (Fig 2.9). Superoxide radical anions are mainly produced by NADPH oxidase even though other oxidases such as xanthine oxidase, can generate them and due to their deleterious effect on important molecules of the cells, they are detoxified (Li *et al.*, 1995).

2.3.1.3 Catalase

Catalase (CAT) is an enzyme which contains iron and primarily found in peroxisomes, and also involved in the detoxification of hydrogen peroxide through a catalytic reaction to produce water and O_2 (Mates, 2000). Even though the H_2O_2 produced by SOD is not a radical, it is still recognized as deleterious, due to its likely conversion to extremely reactive hydroxyl radical via Fenton reaction [Fig 2.9] (Wlaschek and Scharffetter-Kochanek, 2005). In addition, catalase can be involved in peroxidative activity where it promotes interaction between hydrogen peroxide and other oxidizable compounds to produce water (Seifried *et al.*, 2007). Hydrogen peroxide has been implicated as the responsible factor in the short life span of neutrophils, however, an investigation by Kasahara and colleagues demonstrated the protective role of catalase in inhibiting spontaneous apoptosis in these neutrophils (Kasahara *et al.*, 1997).

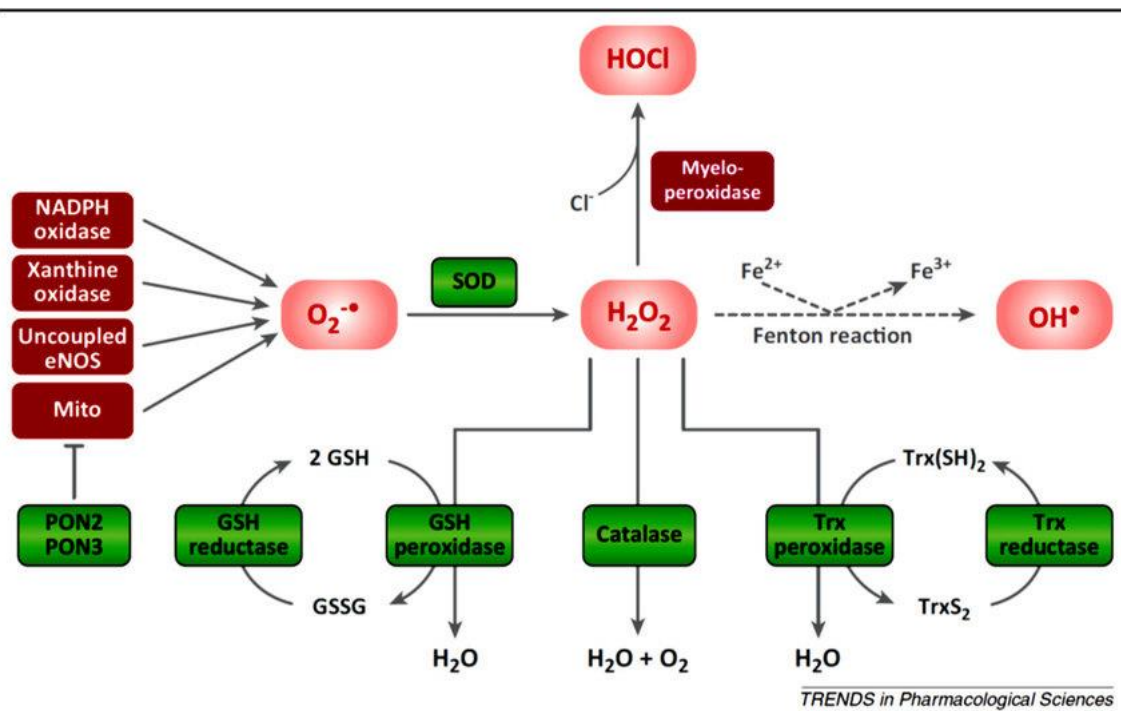


Fig 2.9: ROS-generating and -scavenging enzymes. Leakage of activated oxygen from mitochondria during oxidative respiration results in the generation of is superoxide anion (O_2^-) by NADPH oxidase. Superoxide anion is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 can be converted spontaneously to the extremely toxic hydroxyl radical (OH^\bullet) via Fenton reaction. H_2O_2 can be detoxified by either catalase, glutathione (GSH) peroxidase or thioredoxin (Trx) peroxidase to H_2O and O_2 . Chloride can be oxidized by H_2O_2 to generate the strong oxidizing agent, hypochlorous acid (HOCl), using Myeloperoxidase [MPO] (Kumar, 2017).

2.3.2 Exogenous Antioxidants

Food and medicinal plants such as fruits, vegetables, spices, beverages, cereals, mushrooms, and traditional herbs are the major sources of exogenous antioxidants (Deng *et al.*, 2012). Plant based antioxidants are mostly polyphenols, vitamins (C and E) and carotenoids (Baiano and Del Nobile, 2016). Examples of polyphenols are the flavonoids and lignans which together with carotenoids exhibit numerous biological properties such as antibacterial, anti-inflammatory, antiviral and anti-aging properties (Manach *et al.*, 2004). Irrespective of the protection these plant antioxidants confer to the body, they can act as pro-oxidants if consumed indiscriminately at high dosages (Seifried *et al.*, 2007).

2.3.2.1 Phenolic compounds

Phenolic compounds are naturally found in all plants and belong to a complex group of organic substances. A variety of phenolic compounds are produced and accumulated by higher plants that protects them from tissue injuries and more importantly the damaging effects of free radicals that are the by-products of photosynthesis (Soares, 2002). Phenolic compounds have two classifications namely; polyphenols (sub-classified as tannins and flavonoids) and simple phenolic compounds which are also divided into coumarins and phenolic acids, while phenolic acids are further grouped into either hydroxybenzoic acid (eg vanillic acid, gallic acid, salicylic acid etc) or hydroxycinnamic [eg caffeic acid, ferulic acid, chlorogenic acid etc] (Fig 2.10).

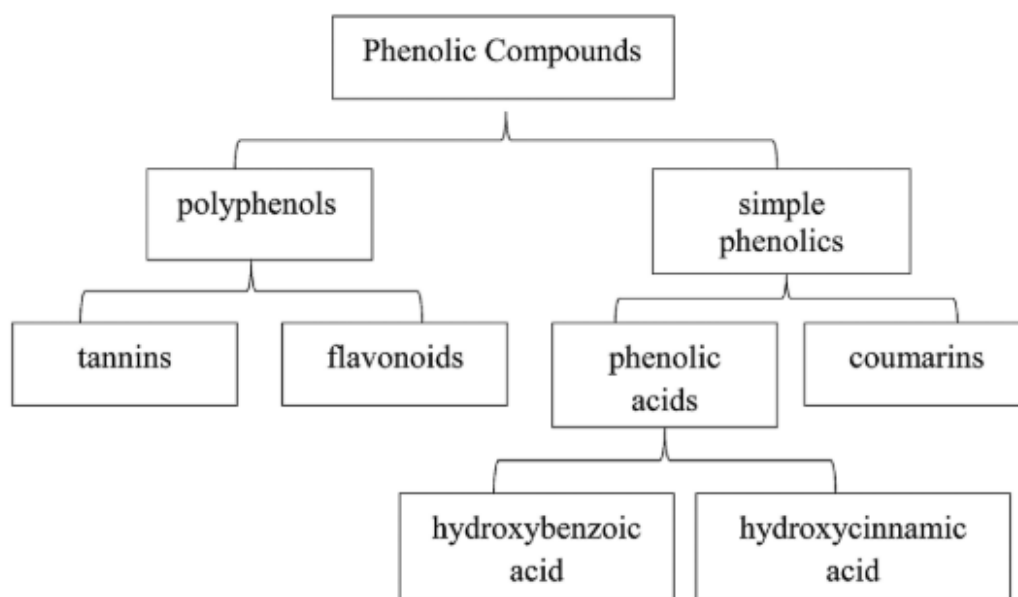


Fig 2.10: Chemical classification of phenolic compounds [Magnani *et al.*, (2014)].

2.3.2.1.1 Gallic acid

Gallic acid (3,4,5-trihydroxybenzoic acid, GA) is a phenolic acid which is found in several plants including wild flowers, fruits and leaves (Ng *et al.*, 2004) with many claims attributed to the health benefits it possesses due to its antioxidant, anticancer, anti-inflammatory, neuro-

protective, analgesic and anti-diabetic properties (Mansouri *et al.*, 2014). The treatment of wounds with natural products has existed from time immemorial and continues to attract keen interest due to the purported safety of these natural products and their easy accessibility hence their use in combating several skin pathologies (Korkina *et al.*, 2012). Several chronic skin diseases such as vitiligo and psoriasis have been treated with polyphenols which are key components of traditional medicines through topical applications to enhance wound healing and exert anti-inflammatory effects (Korkina *et al.*, 2007).

A study by Yang and colleagues demonstrated GA as a potential antioxidant involved in the direct up-regulation and expression of antioxidant genes. They again proved the migratory effect of GA on keratinocytes and fibroblast (Yang *et al.*, 2016). Factors such as extracellular signal-regulated kinases (Erk), focal adhesion kinases (FAK) as well as c-Jun N-terminal kinases (JNK) which are known hallmarks of wound healing have been shown to be activated by GA, demonstrating the beneficial effect it has on wound repair, thus a potentially remarkable wound healing agent (Yang *et al.*, 2016).

2.3.2.2 **Kombucha tea**

Kombucha tea (KT) is sugared tea that has been fermented by a Symbiotic Colony of Bacteria and Yeast (SCOBY) to form a jelly-like membrane (mat) on the tea surface when exposed to oxygen and conducive temperature. Usually, sub-culturing is done every 7-14 days where 10 % of previous ferment is added to a freshly prepared sugared-black or green tea, which then multiplies continuously over time to spread over the surface of the tea entirely before thickening (Dutta and Gachhui, 2007). The two well-known traditional substrates used in Kombucha tea preparation are green and black teas (Gaggia *et al.*, 2019) both of which have been proven to exhibit good antioxidant activity most especially green tea Kombucha (Fu *et al.*, 2014). Kombucha tea has gained popularity for its acclaimed benefits of possessing antioxidant as well as antimicrobial properties (Jayabalan *et al.*, 2008; Sreeramulu *et al.*,

2000) and has several constituents ranging from polyphenols, amino acids, active enzymes, several organic acids as well as many other compounds that form during the fermentation process (Jayabalan *et al.*, 2007).

2.4 TRANSCRIPTIONAL REGULATION OF ENDOGENOUS ANTIOXIDANTS

A myriad of genes that encode antioxidant proteins and detoxifying enzymes have been shown to be triggered by their exposures to antioxidants and xenobiotics, thereby, offering protection against oxidative stress. An array of genes targeting Nrf2, that play a key role in redox homeostasis have been classified as antioxidant response elements (AREs), as these AREs mainly hold the key to transcriptional regulation of cell protective genes (Raghunath *et al.*, 2018). The use of computational analysis have proven that, transcriptional factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), Activating Protein 1 (AP-1), Activating Protein 2 (AP-2), Specificity Protein 1 (Sp1) and CCAAT-Enhancer-Binding Proteins (C/EBP) possess binding sites for the proximal promoter regions of *SOD* genes, thus inducing their expression (Miao and Clair, 2009).

For the positive regulation of catalase expression, transcription factors such as Nuclear Factor Y (NF-Y), Sp1 as well as Forkhead box O3 (FoxO3a) have been described to bind to the core promoter of the *CAT* gene. The activity of FoxO3a has also been proven to be modulated by the protein Akt/PKB (Protein Kinase B) in the PI3K (Phosphoinositide 3-kinases) signaling pathway that eventually promote the expression of the *CAT* gene (Glorieux *et al.*, 2015). The regulation of *GPXI* gene transcription has also been described to be triggered by oxidative mechanisms via NF κ B and AP-1 sites in its promoter region. This was observed in a study by Zhou *et al.* (2001) which indicated an oxidant-induced overexpression of *GPXI* gene in fibroblasts. The regulation of the *GPx-1* promoter at AP-1 sites have also been proposed to involve a number of nuclear factors such as c-fos and c-jun, which are known to be redox sensitive (Abate *et al.*, 1990).

2.5 ACTIVITIES OF EXPRESSED ENDOGENOUS ANTIOXIDANT ENZYMES

The exposure of cells to oxidative stress usually results in the increased expression of antioxidant enzymes to compensate and protect the cells from damage that may be induced by free radicals. The free radicals are normally produced excessively and overwhelm the protective effect of the antioxidants; thus, the expressed endogenous antioxidants are unable to counteract the deleterious effects of the ROS. It has been demonstrated that, the gene expression or activity of antioxidant enzymes are sometimes diminished when exposed to extreme levels of oxidative stress conditions. This led to the hypothesis that, moderate levels of toxic reactants increase antioxidant enzyme expression while high levels of toxic reactants rather reduce the activity of antioxidant enzymes (Gechev *et al.*, 2002). Treatment of wound sites with antioxidants such as curcumin has been shown to increase activity levels of SODs, catalase and glutathione peroxidase to promote wound healing through collagen modulation and ROS level reduction ((Panchatcharam *et al.*, 2006).

In a study by Galán *et al.* (2001), it was demonstrated that pre-incubation of human promonocytic cells (U-937) with antioxidants (N-acetyl-L-cysteine, butylated hydroxyanisole) was able to suppress apoptosis induction when the cells were subjected to stress inducers such as cadmium and X-rays. Another study has proven that during the different stages of wound repair, different ROS- dependent enzymes are expressed where there is up-regulation of SOD1 and SOD2 mRNA levels to orchestrate healing, with the early inflammatory stage observed to have the highest expression (Steiling *et al.*, 1999). High levels of ROS at wound sites has been shown to inhibit the enzymatic activities of the different types of SODs (Vessey and Lee, 1993) which is consistent with the hypothesis that impaired wound healing is associated with reduced SODs activities (Rasik and Shukla, 2000).

2.6 WOUND HEALING

The skin being the largest organ of the body comprises of the outer epidermis, underlying connective tissue, and dermis. It mediates the external and internal environment of the body and therefore regulates temperature, metabolism, immunity and confers protection (Bryant and Nix, 2015). It defends the body from external harm such as pathogen invasion, oxidative stress or UV and as such any damage to the skin requires immediate orchestration of repair processes to heal the wound. Wound healing involves four phases of repair processes namely; hemostasis, inflammation, proliferation and re-epithelialization/remodeling [Fig 2.11] (Gurtner *et al.*, 2008). Hemostasis involves the release of clotting factors by platelets at sites of injury to prevent bleeding (Versteeg *et al.*, 2013). Inflammatory phase is characterized with the production of chemokines and cytokines by neutrophils and macrophages that kill and phagocytize pathogens as well as remove cellular debris. During proliferative phase, formation of new blood vessels from endothelial cells occur through a process called angiogenesis (Midwood *et al.*, 2004), fibroblast growth occurs and form interim extracellular matrix by secreting collagen as well as fibronectin (Chang *et al.*, 2004).

Simultaneously, the epidermis undergoes re-epithelialization through the proliferation of epithelial cells such as keratinocytes that overlay the wound bed for new tissue formation (Garg, 2000) whiles myofibroblast stimulate wound contraction. During maturation and remodelling, realignment with collagen is done and surplus cells are removed via apoptosis (Midwood *et al.*, 2004). Healthy individuals have a higher efficiency of restored epithelium relative to the underlying dermal layers which do not get regenerated perfectly, resulting in the formation of scars, thus affecting the original architecture and function of the tissues. Scar formation is also a common characteristic during wound healing in people with Buruli ulcer disease (Aujoulat *et al.*, 2003). Due to the disruption of this well-ordered process of wound healing by mycolactone, the expected time frame for the wound closure is delayed resulting

in the development of chronic wounds which are public health concern (Harding *et al.*, 2002). To ensure regeneration of wounded tissues and prevent secondary infection of these chronic wounds (Gurtner *et al.*, 2008), proper wound healing is essential therefore necessitating use of wound healing agents.

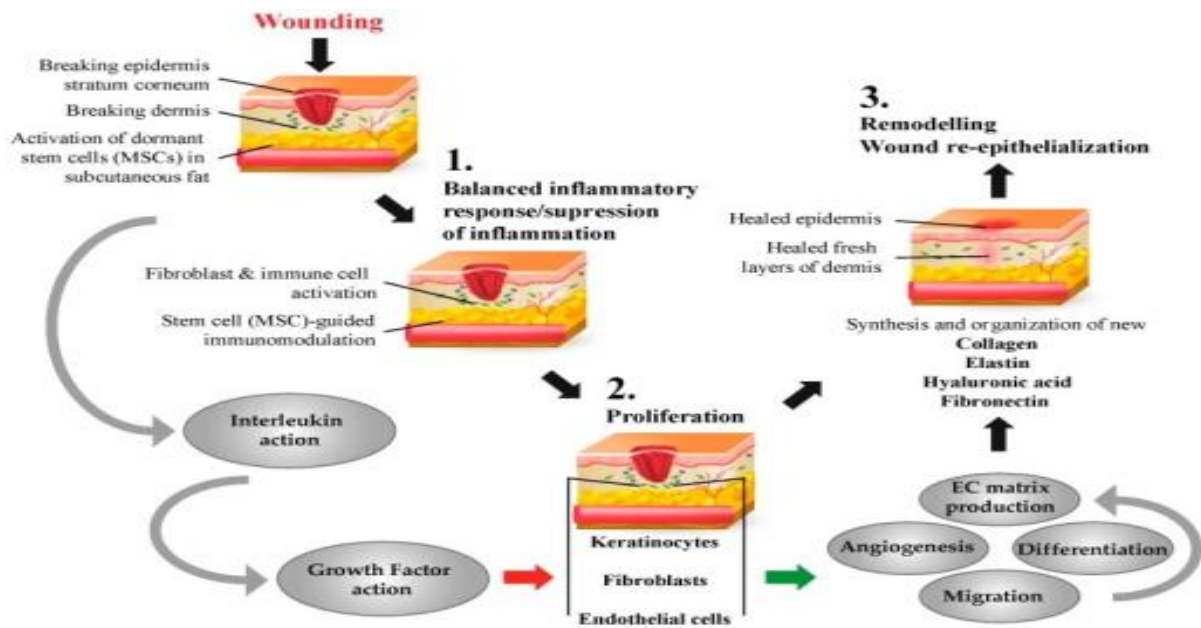


Fig 2.11: The phases of wound repair processes (www.inovanewsroom.org)

2.6.1 Role of Macrophages in Wound Healing

Macrophages, aside their powerful role played in innate immune system, are also important in tissue repair and regeneration, embryonic development as well as clearance of cellular debris (Wynn *et al.*, 2013). Depending on the external cues or microenvironment of the macrophages, they can be polarized to two phenotypes *in vitro*; which are the classically activated macrophages (M1) and alternatively activated macrophages (M2). The M1 macrophages exert pro-inflammatory properties by producing cytokines and microbicidal molecules against pathogens and phagocytizing them, all of which are geared towards host-defense (Mills *et al.*, 2000). The polarization of macrophages towards M1 phenotype has been associated with early phase infection with mycobacterial species such as

Mycobacterium tuberculosis (Chacón-Salinas *et al.*, 2005), *Mycobacterium avium* and *Mycobacterium ulcerans* (Benoit *et al.*, 2008). Macrophages with M2 phenotypes have been subdivided into two groups namely; wound healing macrophages and regulatory macrophages, with the latter having anti-inflammatory properties. These anti-inflammatory properties help resolve the inflammatory phase by producing immunosuppressive cytokine such as IL-10. Wound-healing macrophages on the other hand, promote wound repair by secreting IL-4 and up-regulating the activity of arginase, which is involved in the production of collagen, proline and polyamines for the regeneration of damaged tissues (Mosser and Edwards, 2008).

2.6.2 Effect of Excessive ROS on Wound Healing

Reactive oxygen species play a significant role in host defense against invading pathogens (Clark, 1994) and have recently been demonstrated to be involved in mediating intracellular signaling at low levels (D'Autréaux and Toledano, 2007). A study by Roy *et al.* (2006) demonstrated that wound angiogenesis is enhanced at low levels of hydrogen peroxide. It is reported that regulation of redox is critical in various aspects of wound healing due to recent discoveries that have demonstrated that almost all cells involved in the wound healing process have certain specific enzymes that utilize oxygen to produce reactive oxygen species (Gordillo and Sen, 2003). While low levels of ROS are essential for effective wound healing (Rodriguez *et al.*, 2008), excessive production of ROS can cause detrimental effects on wound healing due to induced oxidative stress (Ponugoti *et al.*, 2013). *In vivo* experiments have demonstrated impairment of wound repair processes in the presence of sustained elevated ROS thus leading to chronic non-healing wounds (Schafer and Werner, 2008). At the molecular level, excessive ROS has also been shown to degrade proteins of the extracellular matrix directly or indirectly, and, therefore, impair the function of dermal fibroblast and keratinocytes (Moseley *et al.*, 2004).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

Synthetic mycolactone A/B (0.1 mg/mL ampule) was acquired as a kind gift from Chemistry department, University of Ghana and the antioxidants; gallic and ascorbic acids were purchased from Sigma Aldrich, USA. The culture medium, Dulbecco's Modified Eagle Medium (DMEM) was also purchased from Gibco® (Thermo Scientific, USA). The cell line RAW 264.7 macrophage (RIKEN BioResource Centre Cell Bank, Japan) was a kind gesture from Prof. Regina Appiah-Opong whilst Kombucha starter culture was provided by Dr W.S.K Gbewonyo of the department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon.

3.2 METHODS

3.2.1 Preparation of Mycolactone Working Concentrations and Antioxidants (Ascorbic acid, Gallic acid and Green tea Kombucha)

3.2.1.1 Preparation of mycolactone working concentrations

Stock solution of mycolactone A/B (0.1 mg/mL), stored at -20°C in the dark was diluted with culture medium to obtain the varying concentrations (0.01-1037 ng/mL).

3.2.1.2 Ascorbic and gallic acid preparation

Stock solution of gallic acid ($1 \times 10^6 \mu\text{M}$) and ascorbic acid ($1 \times 10^5 \mu\text{M}$) were prepared using dimethyl sulfoxide (DMSO) and water, respectively. Working solutions were also prepared from the stock using culture media. The final concentration of DMSO used was 1 %.

3.2.1.3 Preparation of Kombucha tea

Kombucha tea (KT) was prepared as described by Abass *et al.* (2018). A one-litre solution of sucrose (approx. 9.63 g/L) was brought to a boil. One Lipton black tea bag (for black tea

Kombucha) and one Ceylon green tea bag (for green tea Kombucha) were infused separately for 2 minutes in the boiled sugared water. The sugared teas were transferred into separate containers and allowed to cool to room temperature and then seeded with 100 mL of a previous ferment containing a 'baby mat' (Symbiotic Colony of Bacteria and Yeast [SCOBY]). The containers were then covered with linen and allowed to ferment for 14 days at room temperature. The fermented teas were filter-sterilized, and filtrates lyophilized by freeze-drying and kept at -20°C.

3.2.2 Determination of Antioxidant Activity of Black and Green Tea Kombucha

3.2.2.1 DPPH radical quenching activity

The antioxidant activity of the KT's was measured using the DPPH radical as described previously by Jayabalan *et al.* (2008). Different concentrations of black tea Kombucha (BTK) and green tea Kombucha (GTK) ranging from 0.625-5 mg/mL at volumes of 200 µL each were prepared. A 100 µL methanolic DPPH solution of 0.5 mM was added to each of the prepared extracts in triplicates and incubated for 20 minutes. Absorbance was measured at 517 nm using Varioskan Lux spectrophotometer. Freshly prepared butylated hydroxytoluene [BHT] (0.0625-1 mg/mL) was used as standard positive control with methanolic DPPH solution as blank. Percentage antioxidant activity was determined as follows:

$$\% \text{ Antioxidant Activity} = \frac{(\text{Absorbance of the blank} - \text{Absorbance of sample})}{\text{Absorbance of blank}} \times 100$$

Absorbance of blank

3.2.2.2 Total phenolic content of BTK and GTK

The amount of total phenolic compounds in both BTK and GTK extracts was determined according to the Folin-Ciocalteu procedure (Singleton and Rossi, 1965) with slight modifications. A volume of 200 µL of both BTK and GTK extracts at varying concentrations ranging from 0.469-15 mg/mL were diluted with 0.4 mL of distilled water followed by

addition of 0.2 mL of 5% Folin–Ciocalteu reagent. Mixture was incubated for 8 min after which 0.4 mL of 7.5% Na₂CO₃ was added. The absorbance of mixture was measured at 765 nm after 2 hours of incubation in the dark at room temperature using Varioskan Lux plate reader (Thermo Scientific, USA). Gallic acid of concentration range of 0.015-0.125 mg/mL was used as standard and the concentration was expressed as micrograms of gallic acid equivalents (GAE) per g of dry weight of extract.

3.2.2.3 Total flavonoid content of BTK and GTK

The flavonoid content in extracts was determined using 100 µl of both BTK and GTK at varying concentrations ranging from 0.938-3.750 mg/mL. A volume of 100 µL of 2% AlCl₃ was then added followed by 10 min incubation period at room temperature. The absorbance of mixture was measured at 415 nm using the Varioskan Lux plate reader. Quercetin of concentration range of 0.008-0.125 mg/mL was used as a standard and the concentration was expressed as micrograms of quercetin equivalents (QE) per g of dry weight of extract (Nabavi *et al.*, 2008).

3.2.3 Cell Culture

RAW 264.7 macrophages were cultured in T-25 flask (Eppendorf) containing DMEM (supplemented with 10% FBS, L-glutamine and 100x streptomycin/penicillin) at 37°C in the presence of 5% CO₂. Detachment of cells was done by treating with 0.25% trypsin-EDTA (Gibco, USA), followed by treatment with complete medium to stop the trypsin action.

3.2.4 Cytotoxicity and Cytopathic Assay

3.2.4.1 Cytotoxicity of mycolactone and antioxidant compounds against Raw 264.7 macrophages

To evaluate the cytotoxic concentration of mycolactone and antioxidant compounds (ascorbic acid [ASC], gallic acid [GA] and green tea Kombucha [GTK]), varying concentrations were

prepared and treated against Raw 264.7 macrophages. Briefly, the cells were seeded overnight into 96-well plates (Eppendorf) at $\sim 1 \times 10^5$ cells/mL in DMEM supplemented with 10% FBS, 100X penicillin/streptomycin and 2 g/L NaHCO_3 . Cells were incubated with mycolactone (0.01 ng/mL to 1000 ng/mL) for 24-72 hours at 37°C in 5 % CO_2 . Treatment of cells with antioxidant compounds (ASC [62.5 to 1000 μM]; GA [3.75 to 60 μM] and GTK [0.625 to 20 mg/mL]) was also done for 24-48 hours at 37°C in 5 % CO_2 .

3.2.4.2 Cell viability using Alamar blue assay

Cell viability for each of the treatments (mycolactone and antioxidant treatments) was estimated using Alamar blue assay by adding 10 μL of 500 μM resazurin solution (114.55 g of resazurin sodium salt (Sigma, USA) dissolved in 1 mL phosphate buffered saline) to a 90 μL of fresh media to obtain a final concentration of 50 μM . Incubation was done for 24 hours and fluorescence was measured at excitation and emission wavelengths of 530/590 nm, respectively using the Varioskan Lux plate reader. The inhibition concentration (IC_{50}) values for the mycolactone-treated cells were estimated using the GraphPad prism 7 software.

3.2.5 Measurement of Reactive Oxygen Species (ROS)

RAW 264.7 macrophages at a density of 1×10^5 cells/mL were seeded into a 96-well plate (black) overnight and labelled with 100 μL of 20 μM of fluorescent probe 2'-7'-dichlorodihydrofluorescein diacetate [DCF-DA] (ab113851, Abcam, Cambridge, UK) for 45 minutes at 37°C in the dark to detect cellular oxidation (following the manufacturer's instructions). The medium was decanted, washed with 1X PBS and replaced with 100 μL of 100 and 1000 ng/mL mycolactone-containing medium (10 μL mycolactone: 90 μL media) for 24 to 48 hours. As a positive control, cells were treated with 100 μL of 100 μM tert-butyl hydroperoxide (TBHP)-containing media (10 μL TBHP: 90 μL media) and incubated for 24 to 48 hours. Untreated cells served as negative control and fluorescence (485 nm excitation/535 nm emission) was measured using Varioskan Lux plate reader. One

independent experiment was set up and done in triplicates. The percent of control of ROS was calculated as follows;

$$\text{ROS (\% of control)} = \frac{\text{Fluorescence of treated cells} - \text{Fluorescence of blank}}{\text{Fluorescence of TBHP-treated cells} - \text{Fluorescence of blank}} \times 100$$

$$\text{Fluorescence of TBHP-treated cells} - \text{Fluorescence of blank}$$

3.2.6 Measurement of Superoxide Anions

RAW 264.7 macrophages were seeded at a density of 1×10^5 cells/mL in a 96-well plate (black) overnight. Media was decanted and replaced with 90 μL each of 100 and 1000 ng/mL mycolactone-containing medium followed by the addition of 10 μL of 2.7 mg/mL nitroblue tetrazolium (NBT) solution. Media was decanted after 24 hours of incubation and cells were washed 3 times with PBS to remove any traces of extracellular NBT solution. TBHP-treated cells were used as positive control whilst untreated cells served as negative control. Cells were lysed with 60 μL of 2M KOH to release intracellular formazan whilst 70 μL of DMSO was added to solubilize the released formazan and gently shaken for 10 min at room temperature (Sim *et al.*, 2006). Absorbance was read at 620 nm using Varioskan Lux plate reader. One independent experiment was set up and done in triplicates. The level of superoxide anion (% control) was calculated as follows;

$$\text{O}_2^{\cdot-} (\% \text{ of control}) = \frac{\text{Absorbance of treated cells} - \text{Absorbance of blank}}{\text{Absorbance of TBHP-treated cells} - \text{Absorbance of blank}} \times 100$$

$$\text{Absorbance of TBHP-treated cells} - \text{Absorbance of blank}$$

3.2.7 Determination of Antioxidants Effect on Induced Intracellular Superoxide Anions and Cytoprotection

Superoxide anions detection and cell viability assessment were performed using nitroblue tetrazolium (NBT) assay and Alamar blue assay respectively after the three model of treatments (Pre-treatment, Co-treatment and Post-treatment) were done. Final concentrations of antioxidants used for the treatments were ASC (500 μM), GTK (2.5 mg/mL) and GA (30

μM) and that of mycolactone was 1037 ng/mL. Cells were seeded in a 96 well-plate overnight in 100 μL growth medium.

For pre-treatment of cells, medium was decanted and replaced with 90 μL of fresh medium and 10 μL of antioxidants. Incubation was done for 24 hours at 37°C in the presence of 5% CO_2 after which antioxidant-containing media was decanted. Cells were washed with PBS twice to remove any traces of antioxidants and finally replaced with mycolactone-containing media (90 μL fresh media and 10 μL of mycolactone) and further incubated for 24 hours. Cells were washed with 1X PBS twice, and replaced with either fresh 50 μM Alamar blue-containing media (90 μL media and 10 μL of Alamar blue solution) or 0.27 mg/mL NBT-containing media (90 μL media and 10 μL of NBT solution) for 24 hours to determine cell viability and superoxide anions, respectively.

In the co-treatment model, the cells were cultured with 80 μL of media, 10 μL of the antioxidants and 10 μL of mycolactone concurrently and incubated at 37°C in the presence of 5% CO_2 for 24 hours. Cells were then washed with 1X PBS twice to remove traces of both antioxidants and mycolactone and replaced with either fresh Alamar blue- or NBT-containing media for 24 hours.

Post-treatment model involved prior treatment of the cells with mycolactone-containing media (90 μL media and 10 μL mycolactone) for 24 hours, washing of cells with 1X PBS to remove traces of the mycolactone and subsequently, the addition of the antioxidants for another 24 hours. Cells were again washed with 1X PBS twice to remove traces of the antioxidants and replaced with either Alamar blue- or NBT-containing media for another 24 hours. Tests for superoxide detections are representative of one independent experiment, done in triplicates. Tests for cell viability are representative of 3 to 4 independent experiments, each done in triplicates.

The percent of control of cell viability was calculated as follows;

$$\text{Cell viability (\% of control)} = \frac{\text{Fluorescence of treated cells} - \text{Fluorescence of blank}}{\text{Mean fluorescence of untreated cells} - \text{Fluorescence of blank}} \times 100$$

$$\text{Mean fluorescence of untreated cells} - \text{Fluorescence of blank}$$

3.2.8 Measurement of Apoptosis and Necrosis

Apoptosis and necrosis detection kit (ab176749, Abcam) was used to detect apoptotic and necrotic cells as described previously (Jung *et al.*, 2018). Briefly, 200 μL of cell suspension was seeded on coverslips at a density of 1×10^5 cells/well in a 24-well plate and treated with mycolactone (1037 ng/mL) for 24 hours. Cells were washed 1-2 times with 100 μL assay buffer and decanted. Fresh assay buffer (200 μL) was then added to the cells and stained with 2 μL of Apopxin green solution which has a high affinity for exposed phosphatidylserine (PS) for the detection of apoptosis. A membrane impermeable dye 7-AAD (7-aminoactinomycin D) which labels the nucleus of damaged cells was also added at a volume of 1 μL to detect necrosis. Untreated cells were also stained with 1 μL CytoCalcein Violet 450, which is a live cell staining dye that stains the cytoplasm.

Stained cells were incubated in the dark at room temperature for 30 – 60 minutes after which cells were washed and dried. Results were then analyzed using fluorescence microscope through the FITC channel which is observed as a green fluorescence (Ex/Em = 490/525 nm) for apoptosis, TRITC channel, observed as red fluorescence (Ex/Em = 546/647 nm) for necrosis and DAPI channel detected as blue fluorescence (Ex/Em = 405/450 nm) for live cells. Cell counting was done using ImageJ software where percentage average number of cells is representative of five different fields, captured at the different channels with the same cell population. One independent experiment was set up and done in triplicates.

3.2.9 Gene Expression Analysis

3.2.9.1 Total RNA extraction

Total RNAs of both treated and untreated RAW 264.7 macrophages at density of $\sim 1 \times 10^5$ cells/mL were isolated using ZymoQuick-RNA MiniPrep Plus Mini kit (Invitrogen), following the manufacturer's instructions. Briefly, cells were lysed and homogenized using 300 μ L of RNA lysis buffer followed by centrifugation at 10,000 g for 30 seconds to clear homogenate and remove majority of gDNA. Supernatant was then used for RNA purification by mixing with equal volume of ethanol (1:1). RNA was sieved through a column by centrifuging at 10,000 g for 30 seconds and treated with 80 μ L of DNase 1 reaction mix for 15 minutes at room temperature (20-30°C) to ensure complete removal of any DNA contaminants. Sieved RNA was washed three times with RNA Prep buffer to ensure complete clearance of DNase 1 reaction mix. RNA was eluted into an RNase free tube using 100 μ L of DNase- and RNase-free water and concentrations were determined using Nanodrop.

3.2.9.2 Quantitative reverse transcription PCR assay (RT-qPCR)

Quantitative Reverse Transcription PCR was performed using Luna Universal One-Step RT-qPCR kit (New England Biolabs), following the manufacturer's instructions. Briefly, an assay mix of all the components was made and well mixed by vortexing. Assay mix was aliquoted into qPCR tubes followed by addition of RNA templates of the various cell treatments. Samples were assayed in triplicate in one run (40 cycles), which was composed of 4 stages, 55°C for 10 min (reverse transcription), 95°C for 1 min for each cycle (initial denaturation), 95°C for 10 seconds (denaturation) and finally the extension step at 60°C for 30 seconds. Gene expression was calculated relative to the endogenous control sample (β -actin) to determine the relative expression values using the $2^{-\Delta\Delta C_t}$ method (where C_t is the threshold cycle). The PikoReal 24 Real-Time PCR System (Thermo Scientific) was programmed with indicated thermocycling protocol and the generated data was analysed.

3.2.9.3 Primer set for PCR

PCR primers used were superoxide dismutase (SOD2), catalase (CAT) and glutathione peroxidase (GPX) as well as β -actin which served as the internal reference primer (control).

Table 3.1 List of primers used for PCR amplifications

Gene	Forward Primer Sequences	Reverse Primer Sequences
SOD2	ATGTTGTGTCGGGCGGCG	AGGTAGTAAGCGTGCTCCCACACG
CAT	GCAGATACCTGTGAACTGTC	GTAGAATGTCCGCACCTGAG
GPX1	AAGGAGGTGCAGGCGGCTGTGAGG	GCGCGGAGAAGGCATACACGGTGG
β -actin	TGGAATCCTGTGGCATCCATGAAAC	TAAAACGCAGCTCAGTAACAGTCCG

3.2.10 Statistical Analysis

Differences between treatments and treatments for combined data were tested for statistical significance using student's t-test, one- way and two-way ANOVA. Statistical significance was determined with $p < 0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 DPPH SCAVENGING ACTIVITY OF BLACK AND GREEN TEA KOMBUCHA

The antioxidant activity of prepared black tea Kombucha (BTK) and green tea Kombucha (GTK) was assessed by determining their scavenging ability against DPPH (Fig 4.1A). Butylated hydroxytoluene (BHT) was used as positive control (Fig 4.1B). It could be observed that antioxidant activity of both teas increased with increasing concentration. A plot of antioxidant activity (%) against concentration was used to extrapolate effective concentration (EC_{50}) of both tea with green tea Kombucha giving an EC_{50} of 2.811 mg/mL whilst that of black tea Kombucha was 3.974 mg/mL. However, these were 5x and 7x respectively less potent antioxidants than BHT.

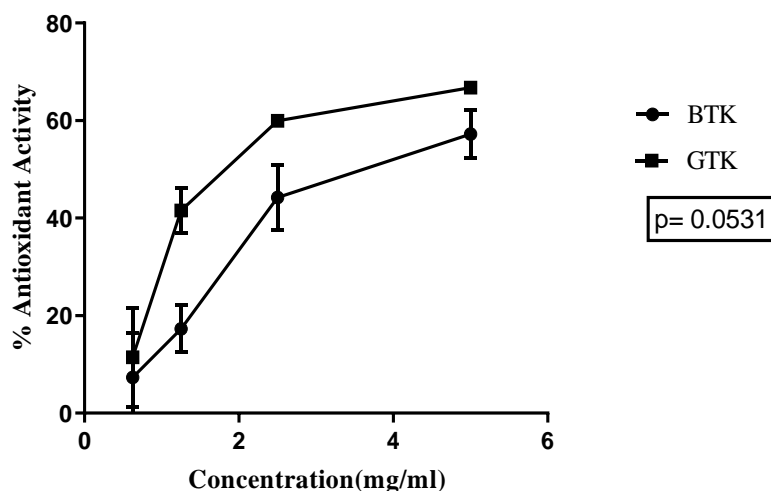


Fig 4.1A: Antioxidant activity of black and green tea Kombucha. Methanolic DPPH solution of 0.5 mM was added to prepared extracts (0.625-5 mg/mL) and incubated for 20 min and absorbance was read at 517 nm. Results are representative of one independent experiment with three replicates (means \pm SEM). BTK= black tea Kombucha; GTK= green tea Kombucha.

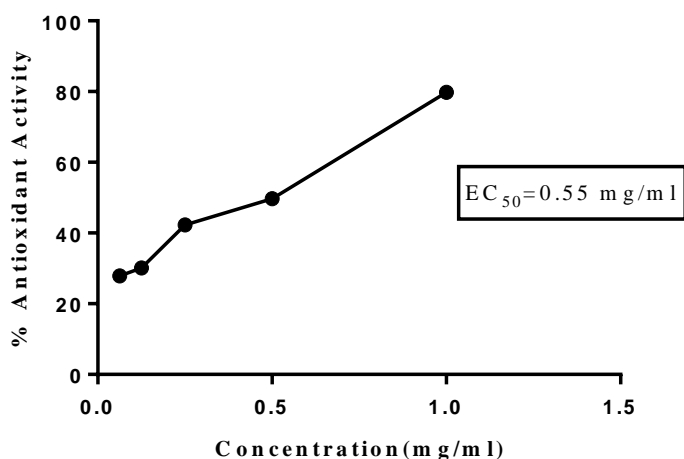


Fig 4.1B: Antioxidant activity of BHT. Methanolic DPPH solution of 0.5 mM was added to BHT solutions (0.0625-1 mg/mL) and incubated for 20 min and absorbance was read at 517 nm. Results are representative of one independent experiment with three replicates (means \pm SEM).

4.2 TOTAL PHENOLIC CONTENT OF BLACK AND GREEN TEA KOMBUCHA

The total phenolic content (TPC) of the black tea Kombucha (BTK) and green tea Kombucha (GTK) was assessed using gallic acid calibration curve (appendix: Fig 1). TPC of both teas increased with increasing concentration (Fig 4.2). At higher concentrations of 15 mg/mL, the TPCs of GTK and BTK were 10.7 and 8.3 expressed as mg/g gallic acid equivalent (GAE), respectively; thus, the TPC of GTK was only 1.2-fold higher than BTK. Statistical analysis confirmed no significant difference in the TPCs of both tea ($p = 0.0835$).

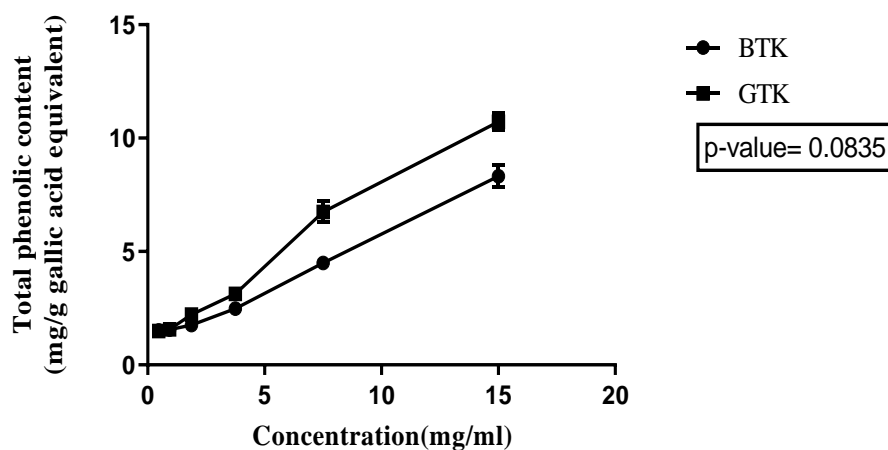


Fig 4.2: Total phenolic content of black and green tea Kombucha. Folin-Ciocalteu reagent (5%) was added to the extracts and incubated for 8 min, followed by addition of 7.5% Na_2CO_3 . Absorbance of the mixture was then measured at 765 nm after 2 hours. Results are representative of one independent experiment with three replicates (means \pm SEM). BTK= black tea Kombucha; GTK= green tea Kombucha.

4.3 TOTAL FLAVONOID CONTENT OF BLACK AND GREEN TEA KOMBUCHA

The total flavonoid content (TFC) of the black tea Kombucha (BTK) and green tea Kombucha (GTK) was assessed using a quercetin standard curve (appendix: Fig 2). TFC of both teas showed no variation even at increasing concentration (Fig 4.3). At higher concentrations of 3.75 mg/mL, the TFCs of GTK and BTK were 0.65 and 0.67 expressed as mg/g quercetin equivalent (QE), which did not differ from the lowest concentrations of both teas (BTK yielded 0.6 mg/g QE and that of GTK was 0.58 mg/g QE). Statistical analysis indicated no significant difference in the TFCs of both tea ($p = 0.06$).

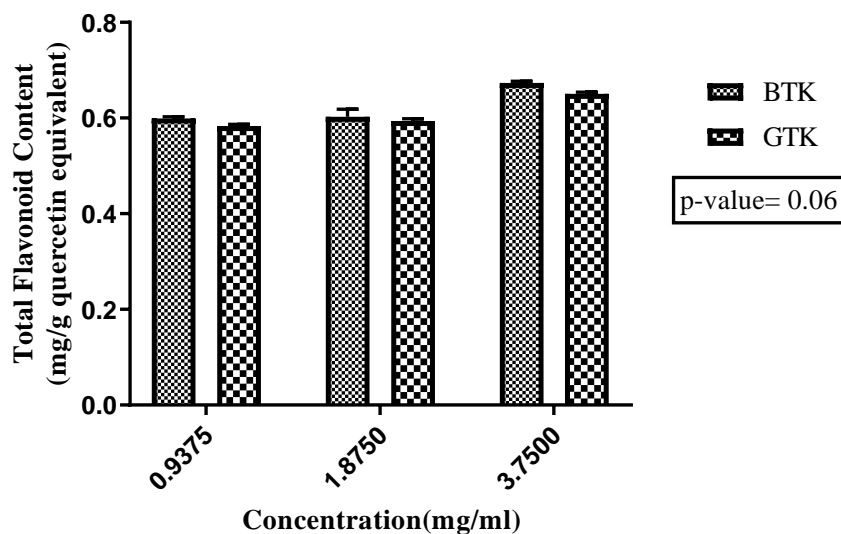


Fig 4.3: Total flavonoid content of black and green Kombucha tea. Two percent AlCl_3 was added to the extracts and incubated at room temperature for 10 min. The absorbance of mixture was then measured at 415 nm using Varioskan Lux plate reader. Results are representative of one independent experiment with three replicates (means \pm SEM). BTK= black tea Kombucha; GTK= green tea Kombucha.

4.4 MYCOLACTONE IS CYTOTOXIC TO RAW 264.7 MACROPHAGES

The cytotoxic effect of mycolactone on RAW 264.7 macrophages was assessed after 24, 48 and 72 hours of incubation at varying concentrations of the mycolactone [0.01 ng/mL to 1000 ng/mL] (Fig 4.4A). Higher concentrations (100-1000 ng/mL) resulted in more than 50% reduction in cell viability while lower concentrations (0.01-10 ng/mL) had no toxic effects. Cell viability decreased with prolonged exposure of cells to mycolactone with cell death being prominent at 48 and 72 hours of treatment. Concentrations of mycolactone that inhibited 50% (IC_{50}) of viable cells were 1037, 35 and 47 ng/mL for the 24, 48 and 72 hours of treatments, respectively (Fig 4.4B). The IC_{50} values were extrapolated from the graph using Graphpad prism 7.

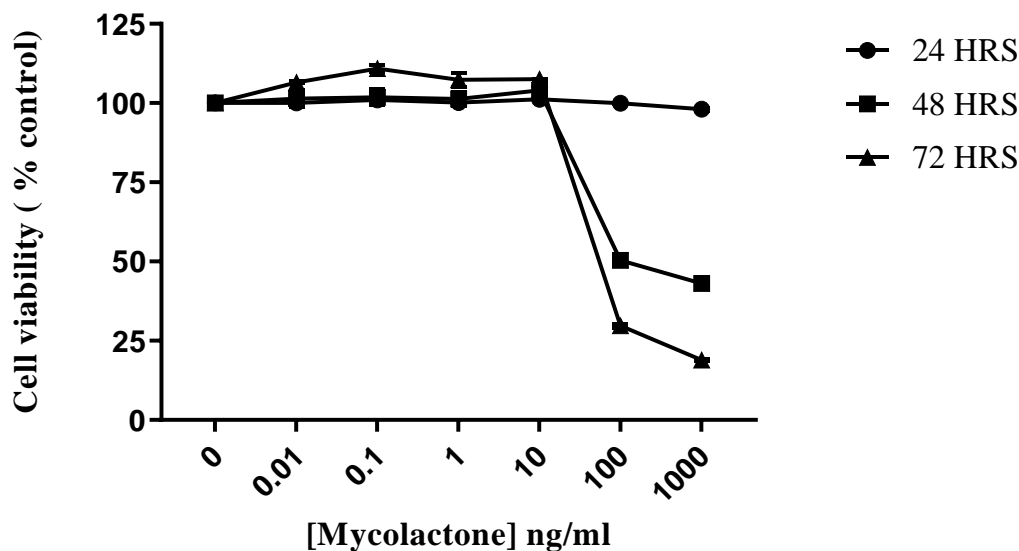


Fig 4.4A: Cytotoxicity of mycolactone against RAW 264.7 macrophage. Cells were treated with mycolactone at varying concentrations and different time points to determine the concentrations at which mycolactone becomes toxic to the cells. Alamar blue assay was used to detect metabolic activity within cells and fluorescence was measured at excitation and emission wavelengths of 530/590 nm. Results are representative of one independent experiment with three replicates (means \pm SEM).

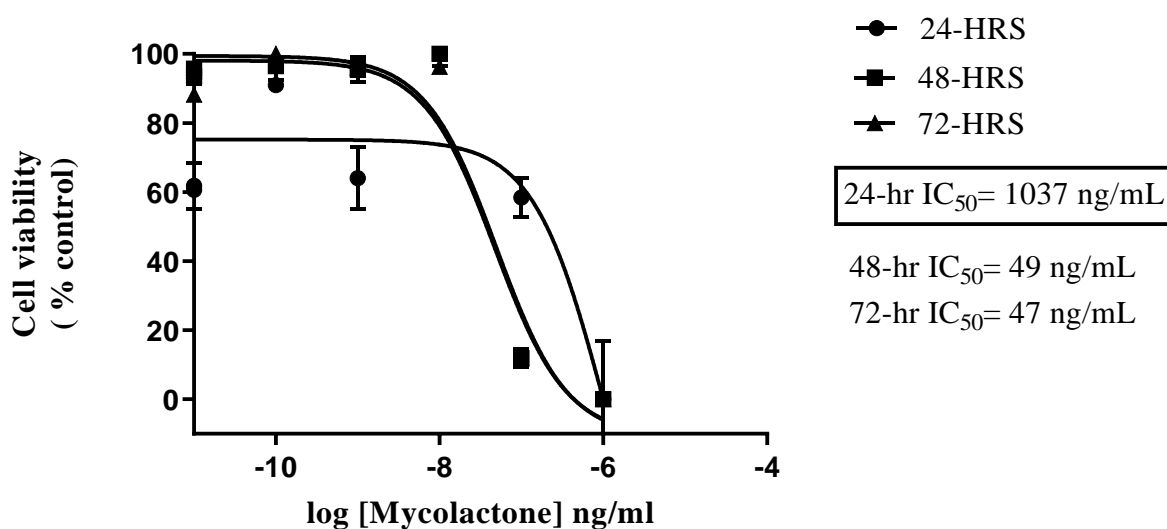


Fig 4.4B: Concentration-response curve for mycolactone-induced RAW 264.7 macrophages. The inhibition concentration (IC_{50}) of mycolactone which causes 50% of cell death was determined at different time points (24, 48 and 72 hours). Results are representative of one independent experiment with three replicates (means \pm SEM).

4.4.1 Cytopathic Effects of Mycolactone

Cytopathic effects were observed when cells were treated with mycolactone (1000 ng/mL) after 24 hours with characteristic cell detachment from monolayer (Fig 4.5 B).

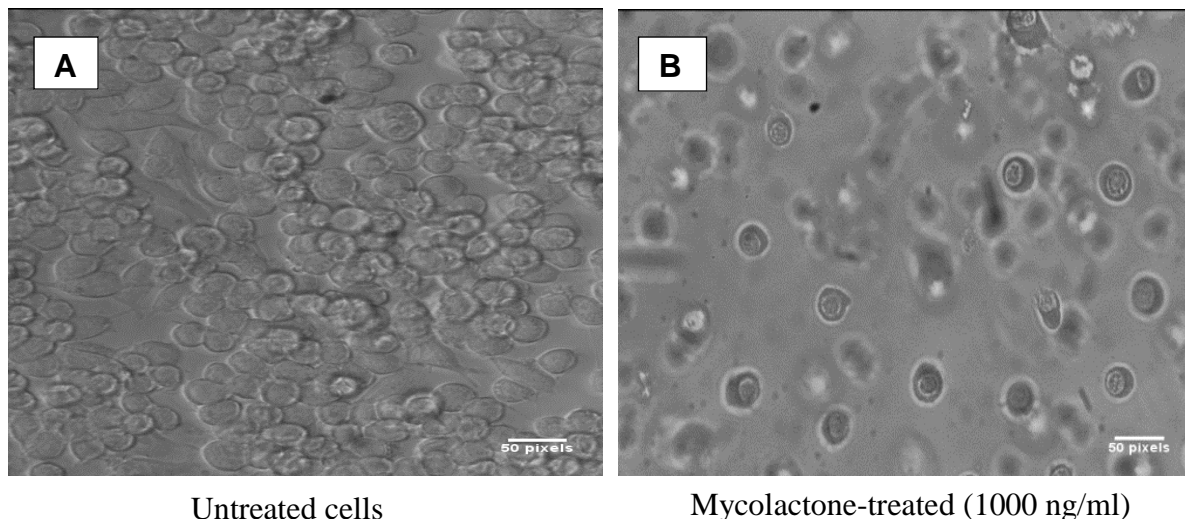


Fig 4.5: Cytopathic effects of mycolactone on RAW 264.7 macrophages. (A) Untreated RAW 264.7 macrophages, (B) RAW 264.7 macrophages treated with mycolactone at 1000 ng/ml for 24 hours. The cells were observed with a light microscope using the X40 objective lens (scale bar = 50 pixels).

4.5 CYTOTOXICITY OF ANTIOXIDANTS ON RAW 264.7 CELLS

Varying concentrations of antioxidants ranging from 62.5 to 1000 μ M ascorbic acid (ASC), 3.75 to 60 μ M gallic acid (GA) and 0.625 to 20 mg/mL green tea Kombucha (GTK) were prepared and used in treating RAW 264.7 macrophages in order to investigate their effect on antioxidant-induced cell death. Concentrations of ASC, GTK and GA above 500 μ M, 2.5 mg/mL and 30 μ M, respectively, decreased cell viability, as the duration of treatment prolonged above 24 hours. Concentrations of the antioxidants that were non-toxic to RAW 264.7 cells were used for the pre-/co- and post-treatment experiments. Based on this, the following concentrations of ASC (500 μ M), GTK (2.5 mg/mL) and GA (30 μ M) were chosen as non-toxic levels for subsequent investigations (Appendix: Fig 3, 4 and 5, respectively).

4.6 MYCOLACTONE INDUCES ROS PRODUCTION IN RAW 264.7 MACROPHAGES

Reactive oxygen species (ROS) and superoxide anions production in RAW 264.7 macrophages were determined using DCFDA (fluorescent probe) and nitroblue tetrazolium (NBT) assay, respectively after 24 to 48 hours of treatment with mycolactone (ML) and tert-butyl hydroperoxide (TBHP). The TBHP (100 μ M), which served as a positive control induced production of ROS maximally up to 100% compared to the untreated controls in both 24 and 48 hours (6% and 10%, respectively). In 24 hours, the cells treated with mycolactone at 100 ng/mL and 1000 ng/mL had a significant increase in the levels of ROS (40% and 59%, respectively), relative to untreated control. Whilst an increase in ROS production was observed between the 100 ng/mL and 1000 ng/mL ML-treated cells, ROS levels showed no significant difference between the 24 and 48 hours durations (Fig 4.6A).

In determining the specific ROS, superoxide anions which are the leading actors of ROS were probed. Levels of generated superoxide anions were no different when the macrophages were treated with mycolactone at concentrations of 100 ng/mL and 1000 ng/mL for 24 hours; generating 79% and 76%, respectively (Fig 4.6B).

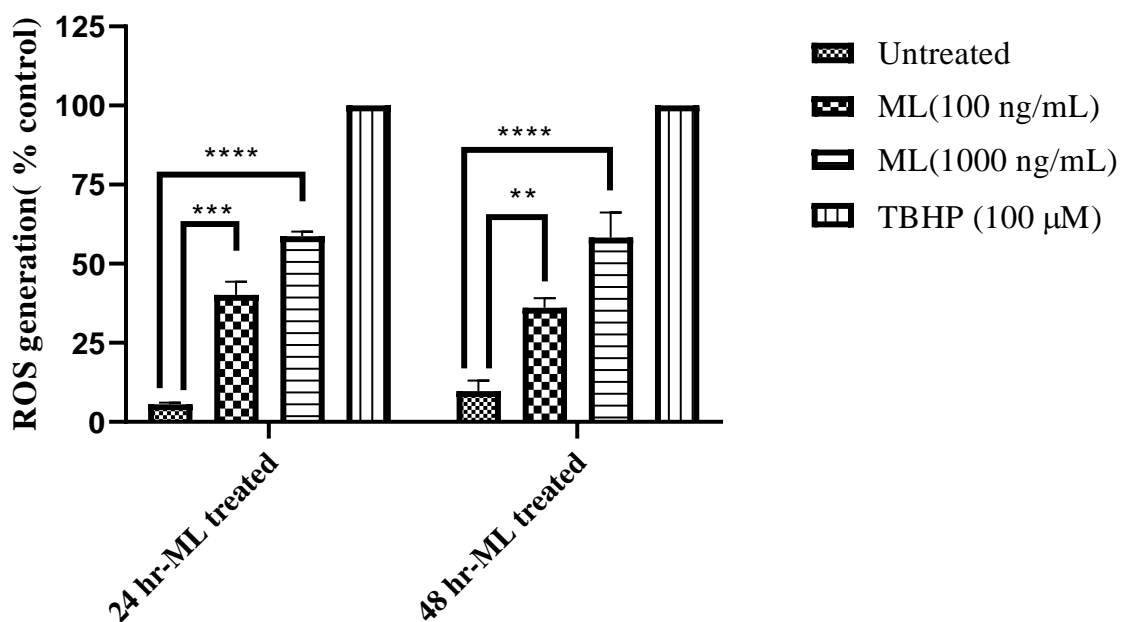


Fig 4.6A: ROS levels in mycolactone-treated RAW 264.7 macrophages. Cells were labelled with DCFDA (20 μM) followed by mycolactone (100 and 1000 ng/mL) treatment for 24 to 48 hours to detect ROS. Fluorescence was measured at excitation and emission wavelengths of 485/535 nm. Results are representative of two independent experiments with 3 replicates (means ± SEM). Values ($p < 0.05$) were considered statistically significant compared to negative control. ([**** $p < 0.0001$]; [*** $p < 0.001$]; [** $p < 0.01$]). TBHP=Tert-butyl hydroperoxide, ML= Mycolactone.

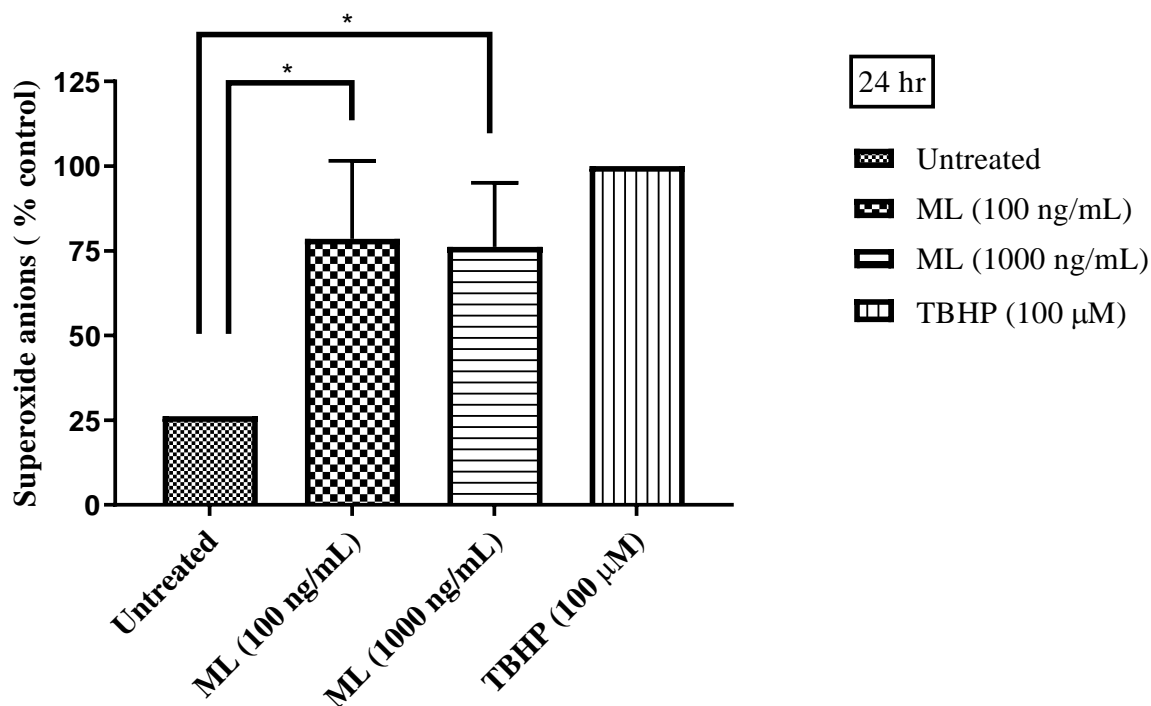


Fig 4.6B: Levels of superoxide anions in ML-treated RAW 264.7 macrophages. Cells were treated with mycolactone (100 and 1000 ng/mL) and NBT solution (0.27 mg/mL) concurrently for 24 hours, cells were lysed to solubilize the superoxide anions for absorbance reading. Results are representative of one independent experiment with 3 replicates (means \pm SEM). P values ($p < 0.05$) were considered statistically significant compared to positive control [$*p < 0.05$]. TBHP=Tert-butyl hydroperoxide, ML= Mycolactone.

4.7 PRE-/CO- AND POST-TREATMENT WITH ANTIOXIDANTS AND MYCOLACTONE.

To assess the mycolactone-induced superoxide anions scavenging ability of the antioxidants, ascorbic acid [ASC] (500 μ M), green tea Kombucha [GTK] (2.5 mg/mL) and gallic acid [GA] (30 μ M) as well as mycolactone (1,037 ng/mL) were used in the three antioxidant-treatment models (Pre-/Co- and post-treatments). Ascorbic acid potently reduced the levels of superoxide anions in all the treatment models with gallic acid also showing significant scavenging effect in the pre and post-treatment experiments, relative to the untreated cells (negative control).

Green tea Kombucha, however, exhibited poor scavenging activity in pre and co-treatment experiments but was very effective at scavenging the superoxide anions in the post-treatment experiments (Fig 4.7).

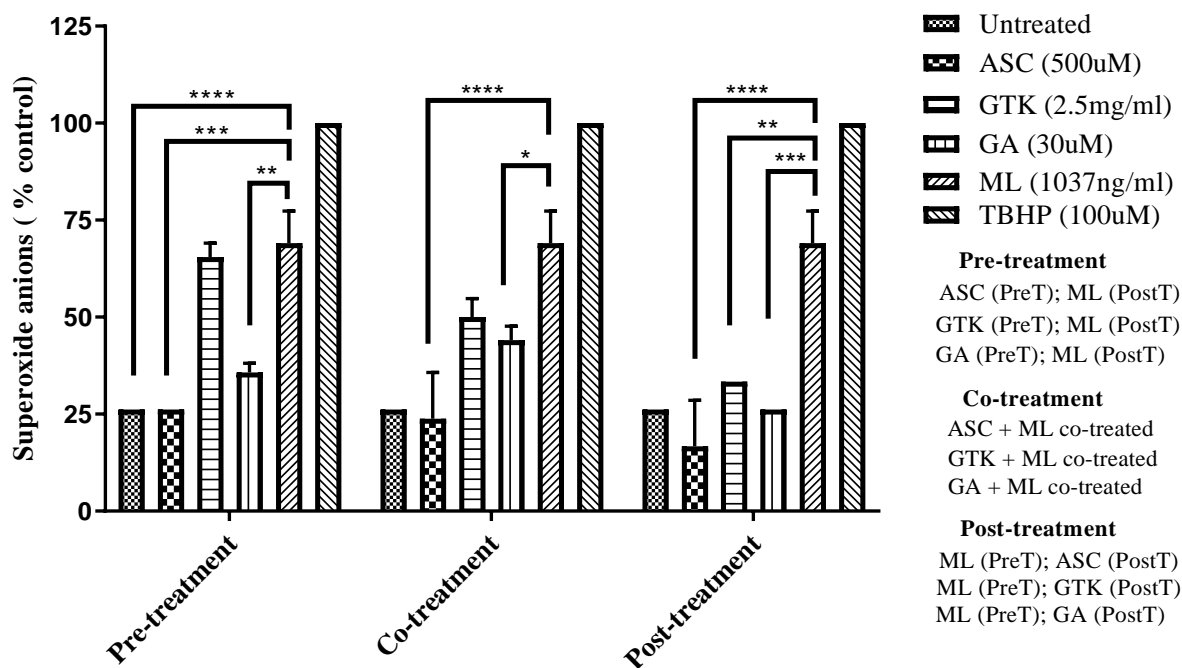


Fig 4.7: Effect of antioxidants on superoxide anion production in RAW 264.7 macrophages.

For the pre-treatment model, the cells were pre-treated (PreT) with the antioxidants (ASC = 500 μ M; GTK = 2.5 mg/mL; GA = 30 μ M.) for 24 hours, washing steps to remove antioxidants, followed by 24 hours mycolactone [ML] (1037 ng/mL) and NBT post-treatments (PostT) and finally, determining superoxide anions. The co-treatment model involved the simultaneous exposure of cells with the antioxidants and the mycolactone as well as the NBT solution for 24 hours, followed by washing steps and finally, determining the superoxide anions. In the post-treatment model, the cells were pre-treated with the mycolactone and the NBT solution for 24 hours, washed to remove traces of the mycolactone and NBT, followed by post-treatment with the antioxidants for another 24 hours. Antioxidants were removed and cells were re-washed before the detection of superoxide anions. TBHP-treated cells served as positive control whilst untreated cells represented negative control. Results are representative of one independent experiment with 3 replicates (means \pm SEM). Values ($p < 0.05$) were considered statistically significant compared to positive control. ([**** $p < 0.0001$]; [*** $p < 0.001$]; [** $p < 0.01$]; [* $p < 0.05$]). TBHP=Tert-butyl hydroperoxide.

4.8 ANTIOXIDANT PROTECTION AGAINST MYCOLACTONE CYTOTOXICITY

4.8 (A) Cell Viability of RAW 264.7 Macrophages in Antioxidant Pre-treatment Model

Based on the superoxide scavenging effects of the antioxidants, it became necessary to investigate whether the antioxidants could protect cells from apoptosis and necrosis. Cell viability assays were performed for the pre-, co- and post-treated experiments. Ascorbic acid showed significant protection of the macrophages when pre-treated with the antioxidants as observed with a cell viability of about 60% compared to the 24 hours ML-treated control cells (44%). However, green tea Kombucha and gallic acid could not cause any significant increase in viability of the cells relative to the 24 hours ML-treated control.

To further investigate the protective effect of the antioxidants, the duration of mycolactone exposure was increased to 40 hours. This surprisingly resulted in a significant reduction in the cell numbers of the ML-treated control compared to the antioxidant-treated (Fig 4.8), whilst the antioxidant-treated cells showed no drastic reduction in cell numbers, relative to the 24 hour ML-post-treated cells.

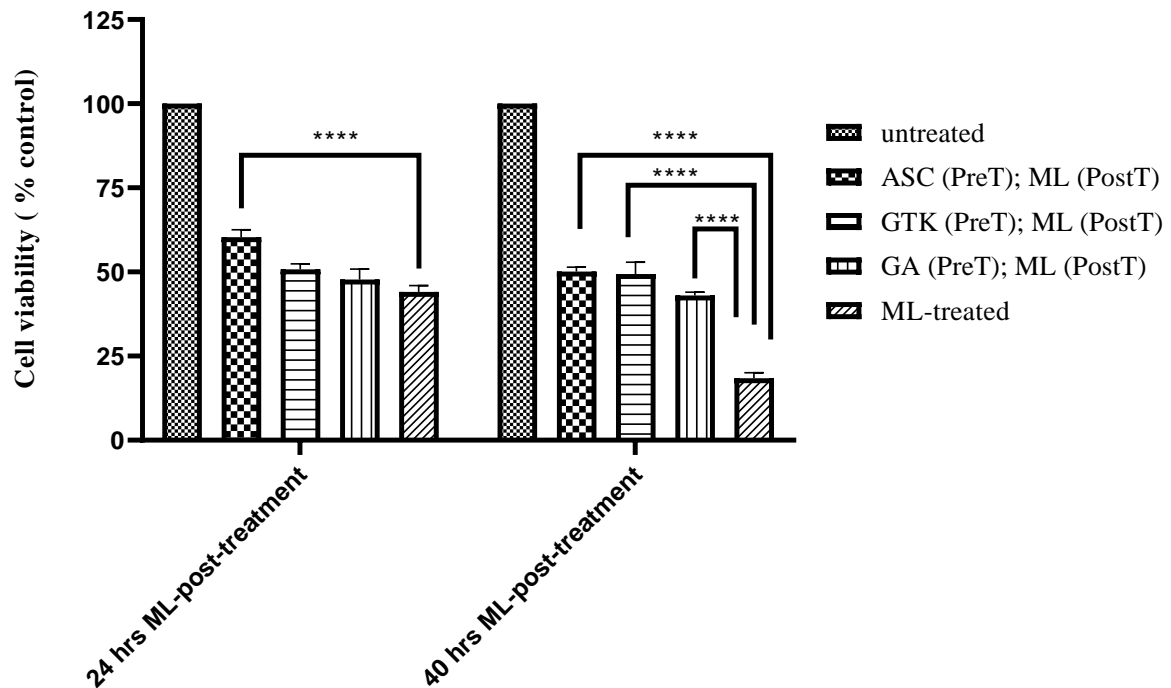


Fig 4.8: Cell viability of RAW 264.7 macrophages after pre-treatment with antioxidants, followed by mycolactone post-treatment. The cells were pre-treated (PreT) for 24 hours with the antioxidants (ASC = 500 μ M; GTK = 2.5 mg/mL; GA = 30 μ M.), antioxidants washed-off, followed by a 24- and 40-hours post-treatment (PostT) with the mycolactone (ML [1037 ng/mL]). Cell viability was determined using Alamar blue assay. Results for 24 hours ML-post-treated cells are representative of four independent experiment with 3 replicates (means \pm SEM), whilst the results of the 40 hours ML-post-treated cells are representative of one independent experiment with 3 replicates. Values ($p < 0.05$) were considered statistically significant compared to mycolactone-treated control. ([**** $p < 0.0001$]).

4.8 (B) Cell Viability of RAW 264.7 Macrophages in Co-/Post-Treatment Model

In the co-treatment experiment, a significant increase in cell numbers was observed when the cells were co-treated with the GTK and mycolactone compared to mycolactone-induced control. There was a non-significant decrease in the cell numbers when treated with both ascorbic and gallic acid (Fig 4.9). In the post-treatment experiments, an interesting observation was made where addition of the antioxidants (ASC, GTK, GA) after the 24 hours of mycolactone pre-treatment, rather, enhanced cell death compared to the 24 and 48 hours ML-treated controls (Fig 4.10).

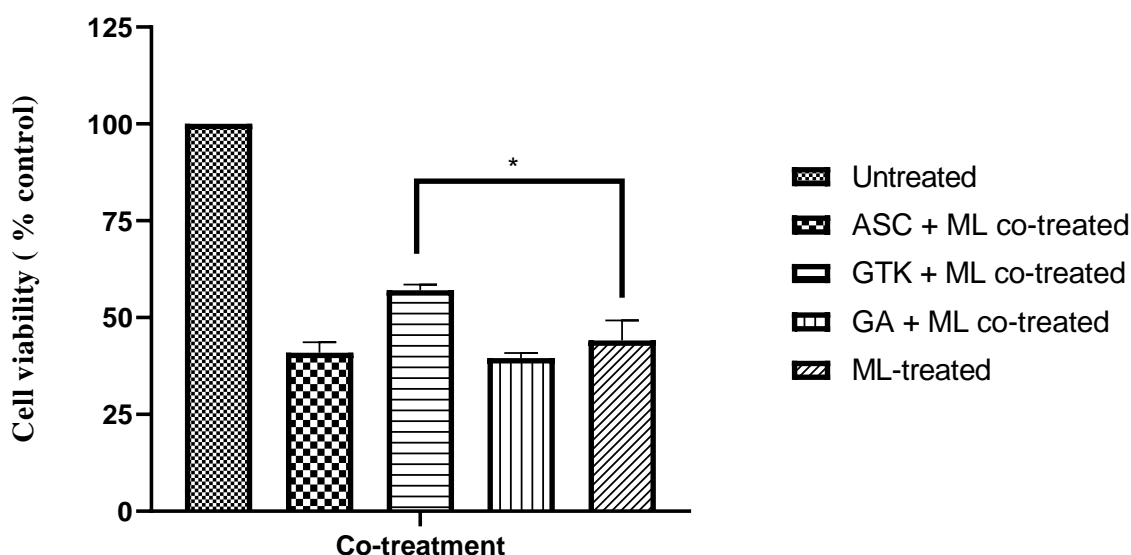


Fig 4.9: Cell viability of co-treated RAW 264.7 macrophages with antioxidants and mycolactone. The co-treatment model involved the simultaneous exposure of cells with the antioxidants (ASC = 500 μ M; GTK = 2.5 mg/mL; GA = 30 μ M.) and the mycolactone [ML] (1037 ng/mL) for 24 hours, followed by washing to remove traces of the antioxidants and mycolactone before the detection of cell viability using Alamar blue assay. Results are representative of three independent experiments with 3 replicates. Values ($p < 0.05$) were considered statistically significant compared to mycolactone-treated control ([* $p < 0.05$]).

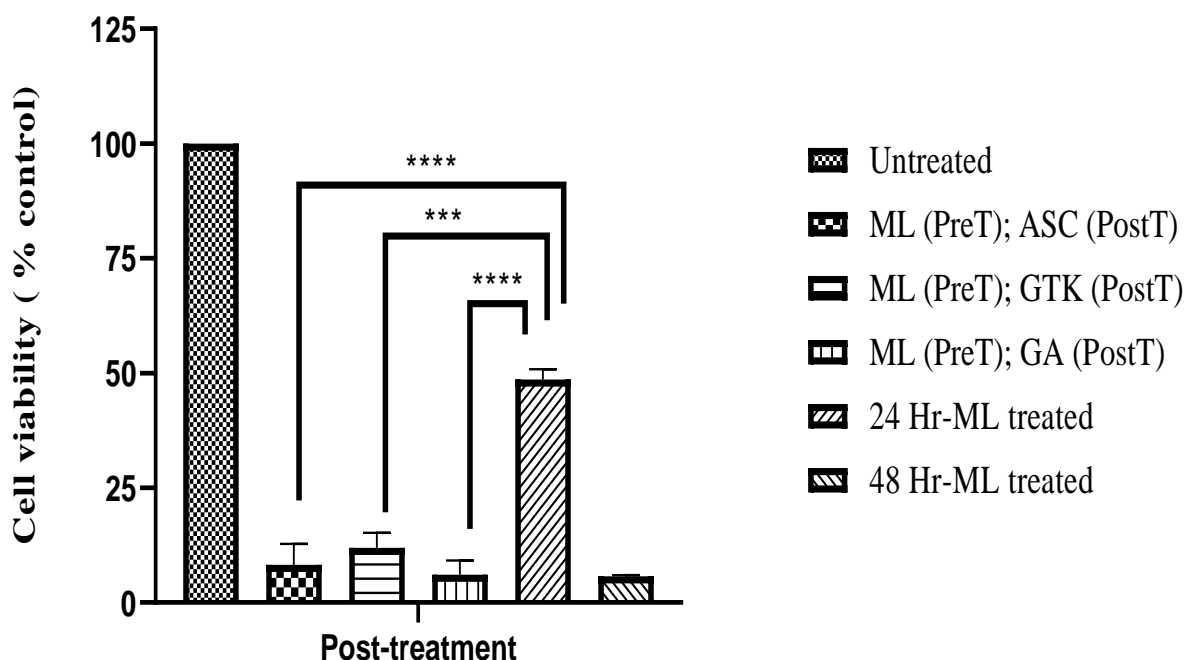


Fig 4.10: Cell viability of post-treated RAW 264.7 macrophages with antioxidants. In the post-treatment model, the cells were pre-treated (PreT) with the mycolactone [ML] (1037 ng/mL) for 24 hours, washed to remove traces of the mycolactone and followed by post-treatment (PostT) with the antioxidants for another 24 hours (ASC = 500 μ M; GTK = 2.5 mg/mL; GA = 30 μ M). Cells were re-washed to remove traces of the antioxidants before the detection of cell viability using Alamar blue assay. Cells were also treated with mycolactone alone at different time points; 24 and 48 hours to serve as controls. Results are representative of two independent experiments with 3 replicates each. Values ($p < 0.05$) were considered statistically significant compared to mycolactone-treated controls ([*** $p < 0.001$]; [**** $p < 0.0001$]).

4.9 MECHANISM OF CELL-DEATH IN MYCOLACTONE-TREATED RAW 264.7 MACROPHAGES

Fluorescent microscopy was used to investigate the nature of cell death induced by the mycolactone (1037 ng/mL) after 24 hours and results showed that apoptosis more than necrosis was the predominant mechanism of cell death (Fig 4.11A and 4.11B). Apoptotic cells with their exposed phosphatidyl serine were stained green by apoxin green indicator whereas late apoptotic/necrotic cells had their nuclei stained red by 7-AAD dye after

mycolactone exposure. Untreated cells had their cytoplasm stained blue with cytochalcein, which represented healthy cells.

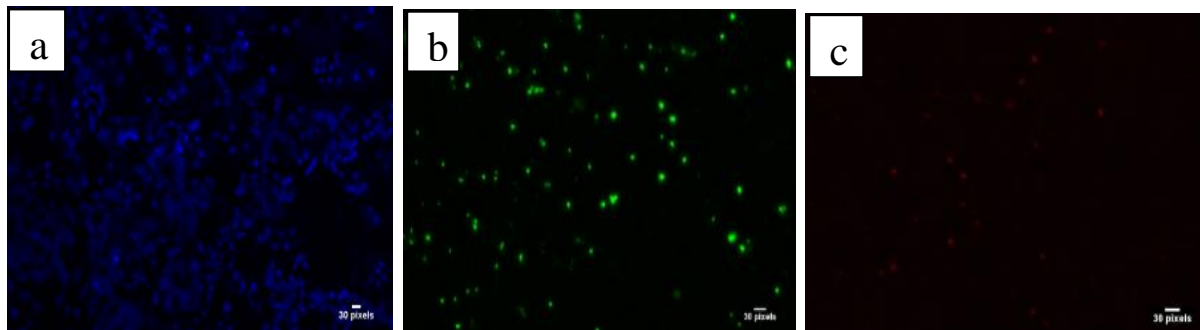


Fig 4.11A: Photographs showing apoptotic and necrotic cell death. RAW 264.7 macrophages were treated with mycolactone at 1037 ng/ml for 24 hours followed by staining to detect apoptosis and necrosis. (a) Untreated cells stained with cytochalcein, (b) Apoptotic cells stained with apoxin green, (c) Late apoptotic/necrotic cells stained with 7-AAD dye. Images were taken with Zeiss fluorescence microscope using the x10 objective lens, through the FITC (green), TRITC (red) and DAPI (blue) channels. Scale bars correspond to 30 pixels.

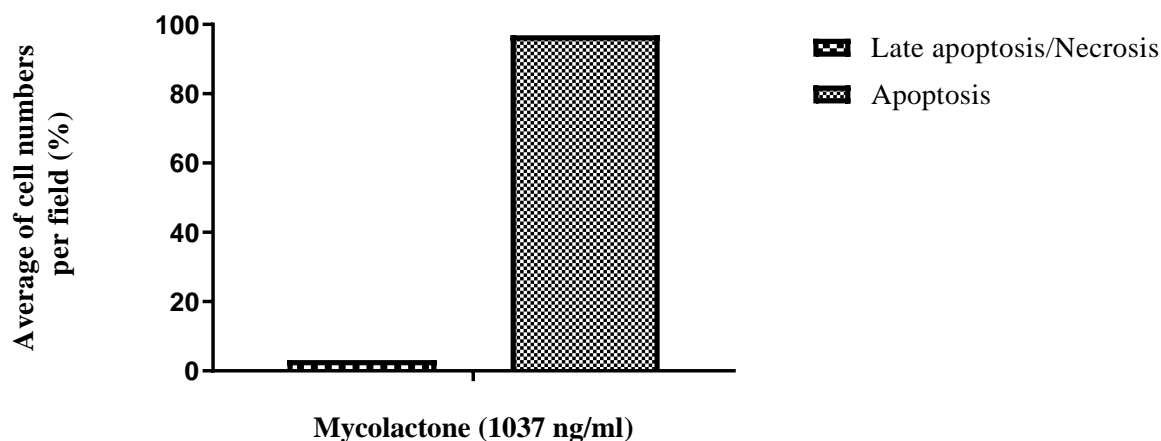


Fig 4.11B: Percentage of average number of apoptotic and necrotic cells. RAW 264.7 macrophages were treated with mycolactone at 1037 ng/ml for 24 hours followed by staining to detect apoptosis and necrosis. Apoptotic cells were stained with apoxin green, while late apoptotic/necrotic cells were stained with 7-AAD dye. Cell counting was done using ImageJ software, where average number of cells expressed in percentage is representative of five different fields, captured at the different channels FITC (green), TRITC (red) and DAPI (blue), with the same cell population.

4.10 EFFECT OF ANTIOXIDANTS ON THE EXPRESSION LEVELS OF ENDOGENOUS ANTIOXIDANT GENES

4.10.1 Antioxidants Enhance Activity of Endogenous Antioxidant Genes in the Pre-treatment Model

The levels of expression of antioxidant enzyme genes (Manganese superoxide dismutase [*SOD2*], catalase [*CAT*] and glutathione peroxidase [*GPXI*]) were determined to investigate the compensatory response mechanism mounted by the cells, to combat the toxic effects of reactive oxygen species (ROS). Here, total transcriptome of ascorbic acid (ASC), green tea Kombucha (GTK) and gallic acid (GA)-treated cells were analysed relative to mycolactone-treated control. *SOD2* expression was found to be up-regulated with a 20- and 18-fold increase in ASC and GTK treated macrophages, respectively. *CAT* expression was predominant in GTK treated cells with a 13-fold increment compared to untreated control, followed by GA-treated cells with a 10-fold. There was an 8-fold up-regulation of *GPXI* with GTK-treated cells and a 5-fold increase with GA-treated cells. Mycolactone-treated cells had moderate expression of the antioxidant enzyme genes with 8-fold increases of *SOD2* and *CAT* whilst *GPXI* was up-regulated to a 3-fold increase (Fig 4.12).

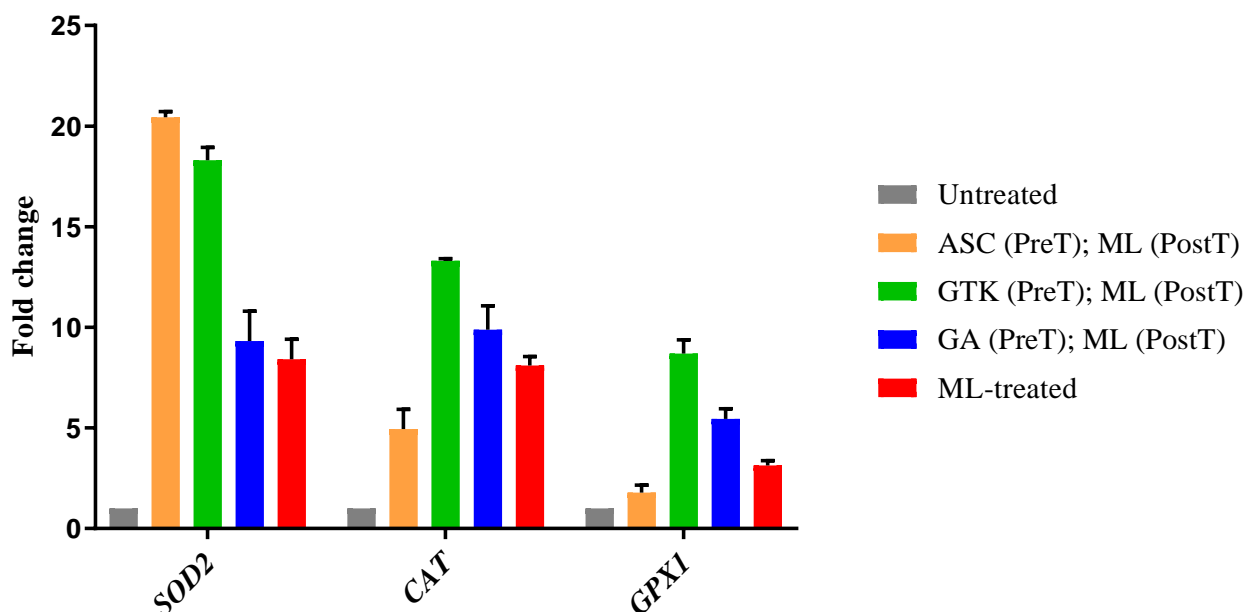


Fig 4.12: Gene expression of *SOD2*, *CAT* and *GPXI* in antioxidant pre-treated and ML-post-treated cells. The cells were pre-treated (PreT) with the antioxidants (ASC = 500 μ M; GTK = 2.5 mg/mL; GA = 30 μ M.) for 24 hours, antioxidants washed-off and followed by a 24-hour post-treatment (PostT) with mycolactone [ML] (1037 ng/mL). RNAs were extracted from both untreated and treated cells and subjected to RT-qPCR to determine the expression levels of *SOD2*, *CAT* and *GPXI*. The total RNA expression profiles were normalized with respect to β -actin. Fold increase of each gene was calculated using the $2^{-\Delta\Delta CT}$ method. Results are representative of one independent experiment with 3 replicates. Fold increase was based on levels obtained for

4.10.2 Antioxidants Enhance Activity of Endogenous Antioxidant Genes in the Co-treatment Model

The co-treatment model which involved the simultaneous treatment of cells with the exogenous antioxidants (ASC, GTK and GA) and mycolactone showed an extremely high expression of the endogenous antioxidant gene catalase (*CAT*) in green tea Kombucha-treated cells with an 800-fold increase. Ascorbic and gallic acid also showed high expressions of *CAT* with 367- and 118-fold increases, respectively. The expression of *CAT* in mycolactone-treated cells was extremely low (8-fold increase compared to untreated cells) compared to the

antioxidant-treated cells, suggesting the enhanced activity of the catalase gene in the presence of the antioxidants. However, expressions of *SOD 2* and *GPX 1* were low relative to *CAT* gene expression in all the exogenous antioxidant-treated cells as well as mycolactone-treated cells (Fig 4.13).

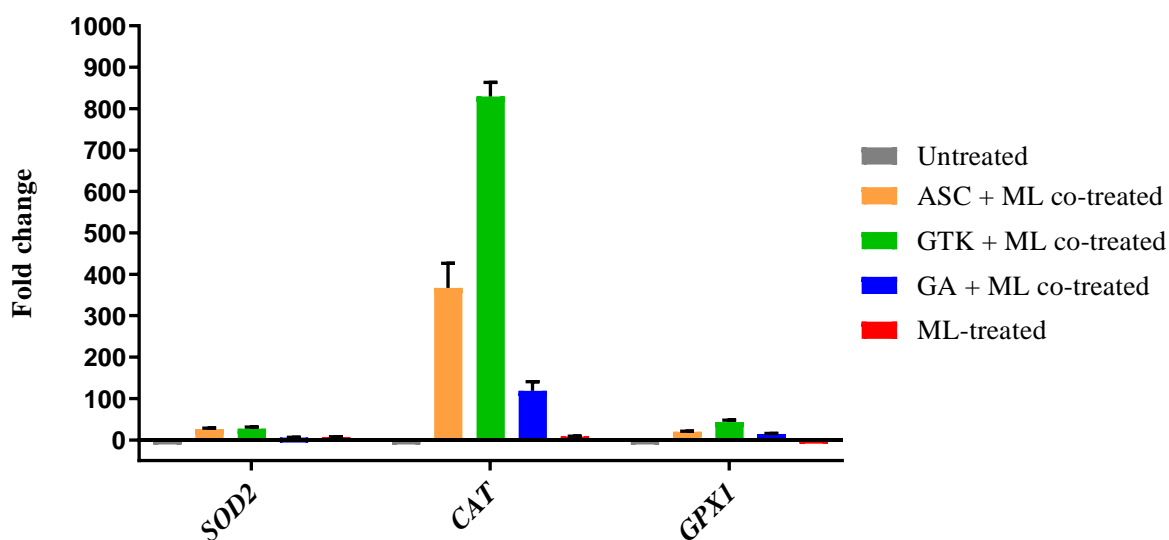


Fig 4.13: Relative gene expression of *SOD2*, *CAT*, *GPXI* in antioxidant-ML co-treatment. The co-treatment model involved the simultaneous exposure of cells with the antioxidants (ASC = 500 μ M; GTK = 2.5 mg/mL; GA = 30 μ M.) and the mycolactone [ML] (1037 ng/mL) for 24 hours, followed by washing to remove traces of the antioxidants and mycolactone. RNAs were extracted from the untreated and treated cells and subjected to RT-qPCR to determine the expression levels of *SOD2*, *CAT* and *GPXI*. The total RNA expression profiles were normalized with respect to β -actin. Fold increase of each gene was calculated using the $2^{-\Delta\Delta CT}$ method. Results are representative of one independent experiment with 3 replicates.

4.10.3 Antioxidants overly Enhance Activity of Endogenous Antioxidant Genes in the Post-treatment Model

The post-treatment model, which involved the pre-treatment of the cells with the mycolactone prior to antioxidants treatments (ASC, GTK and GA), generated several 1000-fold increases in the expression levels of the antioxidant enzyme genes; *SOD2*, *CAT* and *GPXI*, relative to the ML-treated controls. *SOD2* and *CAT* genes were substantially over-

expressed (over 70,000-folds) when the cells were post-treated with ASC and GTK, respectively. The expression of *GPXI* in ASC post-treatment was also hugely expressed with over 45,000-fold increase. While the expression of *SOD2* and *GPXI* had over 24,000-fold increases in GTK post-treated cells, gallic acid treated cells were relatively lower with less than 5000-fold increase (Fig 4.14).

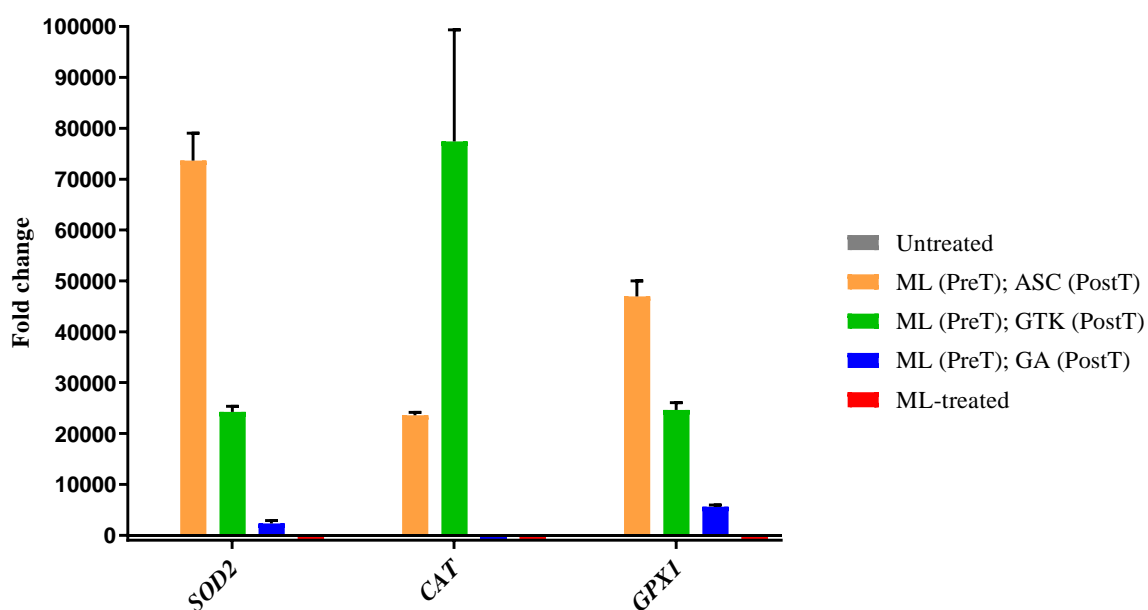


Fig 4.14: Relative gene expression of *SOD2*, *CAT*, *GPXI* in ML-pretreated and antioxidants post-treated cells. The cells were pre-treated (PreT) with mycolactone [ML] (1037 ng/mL) for 24 hours, washed to remove traces of the mycolactone and followed by post-treatment (PostT) with the antioxidants for another 24 hours (ASC = 500 μ M; GTK = 2.5 mg/mL; GA = 30 μ M.). Cells were re-washed to remove traces of the antioxidants. RNAs were extracted from the untreated and treated cells and subjected to RT-qPCR to determine the expression levels of *SOD2*, *CAT* and *GPXI*. The total RNA expression profiles were normalized with respect to β -actin. Fold increase of each gene was calculated using the $2^{-\Delta\Delta CT}$ method. Results are representative of one independent experiment with 3 replicates.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

Background

The cytotoxic effect of mycolactone, the culprit of ulcer formation in Buruli ulcer individuals was studied in RAW 264.7 macrophages in the presence of exogenous antioxidants (ASC, GKT and GA). The scavenging ability of the antioxidants in neutralizing mycolactone-induced reactive oxygen species was demonstrated, the antioxidants up-regulated the expression of endogenous antioxidant enzyme genes. The antioxidants ultimately attenuated the toxic effects of the mycolactone to some extent in the preventive model, hence the macrophage protection. However, the use of the antioxidants in the curative model was the reverse, where cell death was rather potentiated. Our results suggest that, the use of antioxidants as prophylaxis may ensure macrophage survival and in turn enhance proliferation of wound-healing cells such as fibroblast and keratinocytes, to boost wound healing.

Mycolactone is cytotoxic to RAW 264.7 macrophages and induces ROS production

Mycolactone toxicity to most cells, including immune cells was re-affirmed in this study, where toxicity to RAW 264.7 macrophages was observed with increasing concentrations, with no observable differences in the durations used for this study (Fig 4.4A). This is comparable to a study by Simmonds *et al.* (2009) and Hall *et al.* (2014) that reported macrophage sensitivity to mycolactone at high concentrations. The leading actor of ROS; superoxide anions, however, showed no differences in the different mycolactone concentrations used (Fig 4.6B). The apoptotic cell death which was predominantly observed

(Fig 4.11B) is no different from the study by George *et al.* (2000), where J774 mouse macrophage were induced to death via apoptosis after 3 to 5 days of mycolactone exposure.

We then sought to determine whether mycolactone could be inducing ROS production as well as alter the expression of endogenous antioxidant enzyme genes to exert its toxicity to the cells. Different levels of ROS were observed at varying concentrations (Fig 4.6A), which shows similarity to the study by Gronberg *et al.* (2010) that reported a concentration-dependent ROS generation in keratinocytes upon exposure to mycolactone. Moderate levels of toxic substances have been shown to increase antioxidant enzymes gene expression while extremely high levels of toxic substances rather reduce the expression and activity of antioxidant enzymes, in consequence of the harm rendered to the molecular machineries that are essential in inducing the expression of the antioxidant enzymes (Gechev *et al.*, 2002).

In this study, mycolactone at 1037 ng/mL [which was the concentration that inhibited 50% of viable cells] (Fig 4.4B) increased the expression of the antioxidant enzyme genes (*SOD2* and *CAT*) up to 8-folds relative to the untreated macrophages (Fig 4.12). This gives an indication that, the concentration of mycolactone used might not have been toxic enough to down regulate the expression of the endogenous antioxidant enzyme genes in the macrophages. One of the primary factors that regulates the expression and activity of these endogenous antioxidant enzyme genes is dependent on the oxidative status of the cells (Pahl and Baeuerle, 1994) hence, a possible compensatory mechanism initiated by the cells, to quench the effects of the mycolactone-induced ROS. Despite the ROS-resistant characteristics of macrophages, owing to their increased level of ROS reductase and DNA repair proteins during excessive ROS exposure, they are still susceptible to ROS-associated death (Lo *et al.*, 2013). For this reason, it is possible for macrophages which play a key role in the wound repair processes to be subjected to ROS-mediated cell death induced by mycolactone and thus abrogate their stimulatory function on fibroblast and keratinocyte proliferation, which

are also key players in wound healing. This cellular proliferation is promoted by the macrophages' ability to produce numerous growth factors such as TGF- β 1, IGF-1 and VEGF- α (Chujo *et al.*, 2009). In addition, macrophages stimulate fibroblast differentiation to myofibroblast which are involved in the production of extracellular matrix components as well as protein mediators for wound contraction and closure (Murray and Wynn, 2011). In a study by Moseley *et al.* (2004), excessive ROS generation was shown to directly or indirectly degrade proteins of the extracellular matrix and impair the function of dermal fibroblast and keratinocytes, which eventually disrupts wound repair processes, leading to chronic non-healing wounds as demonstrated *in-vivo* by Schafer and Werner, (2008). However, additional function of macrophages has been reported to be the production of extracellular matrix components like fibronectin and collagen of all types, such as type VIII collagen as well as type VI collagen, which are abundantly secreted under certain conditions to modulate cell-cell and cell to matrix interactions (Schnoor *et al.*, 2008). This new dimension of macrophage function can therefore ensure wound healing if macrophages can be protected from ROS-induced cell death and survive to exploit their functions appropriately during wound healing.

Antioxidants induce gene expression and enhance cell viability of ML-treated cells in the preventive model

To determine whether the scavenging ability of the antioxidants was translating into cyto-protection, cell viability assays were done. The use of ascorbic acid in the pre-treatment model (preventive model) was found to increase cell viability (Fig 4.8), which may be attributed to its potent scavenging of the ML-treated superoxide anions (Fig 4.7). This could be considered, a protective effect to avert an imbalance in the antioxidant defense system. Apart from the scavenging role of the ASC, it might have enhanced macrophage protection by up-regulating the expression of the endogenous antioxidant genes *SOD2*, *CAT* and *GPXI* (Fig 4.12). This could be a compensatory mechanism by which the cells are enabled to

counteract the deleterious effects of the superoxide anions, by dismutating superoxide anions to hydrogen peroxide using SOD2. The enhanced expression of the *SOD2*, agrees with a report of Sadi and Güray, (2009), which indicated an increased expression of *SOD2* (Mn-SOD) when ascorbic acid (vitamin C) was used to treat streptozotocin-induced diabetic rats to eliminate the effects of oxidative stress. Up-regulation of the *CAT* gene may also be an indication of accumulated hydrogen peroxide, an end-product of SOD2 dismutation reaction. Glutathione peroxidase 1 (*GPXI*) which also regulates the levels of hydrogen peroxide by catalysing its conversion to water, was moderately expressed (Fig 4.12). Ascorbic acid in the co-treatment model (Fig 4.9) could not protect the macrophages, which can be probably explained by the limited timeframe offered in this simultaneous cell treatment. This perhaps could not allow initiation or induction of any signalling pathway to promote cell survival, hence, the toxicity of the mycolactone being exerted more rapidly than the intervention of the antioxidant.

Green tea Kombucha likewise exerted a protective effect on the cells in the pre-treatment model (Fig 4.8), which is comparable to a study where Kombucha tea was shown to protect hepatocytes from tert-butyl hydroperoxide-induced oxidative stress (Bhattacharya *et al.*, 2011). The antioxidant property exhibited by green tea Kombucha stems from some of its constituents such as polyphenols including flavonoids (Jayabalan *et al.*, 2014) that easily donate electrons to stabilize free radicals to prevent oxidative damage. The presence of phenolic compounds (Fig 4.2) and flavonoids (Fig 4.3) in green tea Kombucha could be responsible for its antioxidant activity (Fig 4.1A). Even though green tea Kombucha has been widely reported as a strong quencher of superoxide anions in cell free systems (Bhattacharya *et al.*, 2011), it could not significantly quench the levels of superoxide anions in the pre and co-treatment models of this study (Fig 4.7), hence its protection could be attributed to factors, other than direct ROS neutralization by the tea. Some of the mechanisms of antioxidant

function have been identified; which includes the prevention of excessive ROS production using endogenous antioxidants and also by repairing ROS-damaged molecules using glutathione (Zhivotovsky and Orrenius, 2011). A study by Ibrahim, (2013) demonstrated a likely protective role of Kombucha tea against oxidative damage by increasing the activities of the endogenous antioxidant enzymes SOD, CAT and reduced glutathione (GSH). The use of green tea Kombucha in the pre-treatment model increased the expression of the *CAT* and *GPXI* genes (Fig 4.12), possibly to moderate the levels of hydrogen peroxide and maintain redox status within the macrophages, which is important in wound healing. This action may be key in the prevention of iron oxidation of the generated hydrogen peroxide to hydroxyl radicals, via Fenton reaction. In a study by Gronberg *et al.* (2010), Fe^{2+} -dependent hydroxyl radicals were implicated as the possible cause of cell death in keratinocytes but the use of an iron chelating compound, deferoxamine, was able to inhibit cell death.

Green tea Kombucha, however, had an increased protection for the cells in the co-treatment model (Fig 4.9), despite its ineffective mop-up of the induced superoxide anions (Fig 4.7), thus a re-affirmation that, other factors could be triggering the tea's macrophage protection. We therefore sought to determine whether the tea could have any extraordinary effect on the expression levels of the *SOD2*, *CAT* and *GPXI*, which could have led to the enhanced protection of the cells relative to the protection in the pre-treatment model. Surprisingly, an exceptionally enhanced level of the *CAT* gene relative to the *SOD2* gene could be observed, which may be ascribed to the complete dismutation of the induced superoxide anions to hydrogen peroxide thus reducing *SOD2* expression, whilst that of *CAT* expression could have been increased due to the build-up of the hydrogen peroxides (Fig 4.13). Again, high expression of the *GPXI* gene might have resulted to GKT's macrophage protection, by reducing lipid peroxides to lipid alcohol to ensure the inhibition of lipid peroxy radicals'

formation, known to cause lipid peroxidation. This action by GPX1 preserves the integrity of cellular and subcellular membranes thus the healthy state of cells.

Gallic acid, as an iron chelator similarly offered protection to the macrophages, though non-significantly in the pre-treatment model (Fig 4.8), which may be due to its observable mop-up of the superoxide anions (Fig 4.7). This agrees with another study in which gallic acid was proven to be a good scavenger of superoxide anions (Masaki *et al.*, 1995). The protection by gallic acid also shows similarity to the study by Gronberg *et al.* (2010), where iron chelating-deferoxamine, was suggested to protect keratinocytes from mycolactone-induced cell death. The cells however declined non-significantly during the co-treatment model, which may be due to an increased cytotoxicity to the cells in the presence of both the mycolactone and the gallic acid, thus losing the beneficial effect. This was expected to lower the expression levels of the *SOD2*, *CAT* and *GPX1* genes, however, the co-treatment model with reduced cell numbers rather yielded higher expressions of the genes relative to the moderate expressions in the pre-treatment model; which ironically had increased cell viability. This may support the premise that, other factors are responsible for the cytotoxicity of mycolactone, other than ROS.

As mycolactone has been previously implicated to induce post-transcriptional effects by inhibiting the translation of some proteins, protein translocation (blockade of Sec61), protein stability and even protein secretion (Simmonds *et al.*, 2009), it is possible that, the transcripts of these endogenous antioxidants, though expressed, may not necessarily be translating to sufficient amounts of proteins to effect their functions. Further studies may be required to ascertain the protein expression levels of these endogenous antioxidant enzymes.

Antioxidants for curative model potentiate death of ML-treated cells in spite of the enormous over-expression of endogenous antioxidant genes

Despite the potent scavenging activity exhibited by the antioxidants in the post-treatment models (Fig 4.7), the addition of the antioxidants (ASC, GTK and GA) rather enhanced cell death (Fig 4.10). The removal of mycolactone from the cells after pre-treatment, followed by post-treatment with non-toxic concentrations of the antioxidants was anticipated to promote proliferation of the cells, however, a stark contrast was observed from the study by Hall *et al.* (2014), which indicated the regain of cell viability and proliferation after removal of mycolactone from the cells, albeit loss of cytokine production. This can be attributed to an already impaired physiological fitness of the cells, induced by the mycolactone, which could have been exacerbated by the addition of the antioxidants. This requires further investigations and therefore, gives an indication that stringent concentration-dependent use of the antioxidants (ASC, GKT and GA) is required for this curative model, either *in vitro* or *in vivo*.

Also, the pro-oxidant nature of ascorbic and gallic acid might have produced this effect depending on their concentration and molecular polarities (Lu *et al.*, 2006). For instance, ascorbic acid which can act as an antioxidant by reducing oxidizing-substances such as hydrogen peroxide (Duarte and Lunec, 2005); can also reduce metals to generate free radicals via Fenton reaction (Stohs and Bagchi, 1995). In this case, both antioxidants could have acted as pro-oxidants by reducing transition metals such as iron and copper, thereby propagating a chain reaction of free radical generation and paradoxically inducing cell death. Also, ascorbic acid could have been converted to its pro-oxidant forms, ascorbyl radicals (ascorbyl palmitate) that might have induced apoptotic cell death through the end-products of lipid peroxidation. However, since the scavenging activity of the antioxidants was effective in the post-treatment model (Fig 4.7), cell death is unlikely to be associated with pro-oxidant

activity of the compounds since pro-oxidants act by either generating ROS or by altering the antioxidant defense system (Halliwell, 2008). Mycolactone-mediated ROS may therefore not be the sole mechanism of cell death hence the increased cell death despite the effective scavenging of the superoxide anions by the antioxidants.

The tremendous over-expression of the antioxidant enzyme genes (Fig 4.14), reiterates the likely negative impact of mycolactone on the post-transcriptional mechanisms within the cells, thus a possible non-translation of the transcripts to proteins or subjection of the antioxidant enzymes to proteosomal degradation, due to blockade of the Sec61 translocon, thus aborting protein modification in the endoplasmic reticulum for subsequent secretion and localization (Simmonds *et al.*, 2009). This might have probably resulted to the woefully reduced cell viability (Fig 4.10), yet enormously enhanced gene expressions of the *SOD2*, *CAT* and *GPXI* after treatments with the exogenous antioxidants (ASC, GTK and GA).

It can also be argued that, the swift cell death could have emanated from the over-expressed antioxidant enzyme genes (*SOD2*, *CAT* and *GPXI*) that might have eliminated basal levels of the ROS required for the physiological functioning of the cells, hence the promotion of apoptosis. This is supported by the studies of Bai and Cederbaum (2000) that associated loss of cellular oxidants to the augmentation of apoptosis in some cell systems. The study showed potentiated apoptosis during catalase overexpression, whilst catalase inhibition rather promoted cell survival. Other studies have also indicated that, the disruption of the physiological oxidant (hydrogen peroxide) signaling by catalase overexpression can lead to decreased activation of the NF κ B survival pathways, hence the inability to counteract an apoptotic pathway (Lüpertz *et al.*, 2008). This to some extent can explain the tremendous cell death.

5.2 CONCLUSION

Mycolactone mediated the production of reactive oxygen species (ROS) in RAW 264.7 macrophages confirming mycolactone as a ROS inducer. The antioxidants used in this study on the other hand, protected the macrophages by scavenging the free radicals induced by mycolactone. If ROS production is a mechanism of mycolactone induced cell death, then the elimination of excessive ROS with antioxidants must be able to ensure macrophage survival to enhance wound healing. The antioxidants beyond their reactive species scavenging roles, induced and enhanced expression of the endogenous antioxidant enzyme genes; superoxide dismutase, catalase and glutathione peroxidase which are first line defense antioxidant enzymes generated to combat the toxic effects of oxygen metabolism. The use of the antioxidants in the protective model, ultimately minimized macrophages' death, hence their role in attenuating the cytotoxic effects of mycolactone. With the enhanced protection of the macrophages as a result of ascorbic acid and green tea Kombucha treatments against the toxicity of mycolactone, it can be deduced that macrophage survival can provide great benefit in wound healing to accelerate growth and proliferation of fibroblasts and keratinocytes which serve as the bedrock in wound healing. If results of this *in vitro* study can be replicated *in vivo*, then ascorbic acid and green tea Kombucha may serve as prophylactic agents that can offer protection for people living in Buruli ulcer endemic areas by preventing or delaying ulcer formation and possibly, enhance wound healing during infection with *Mycobacterium ulcerans*. The use of the antioxidants for curative purposes during MU infections may require further investigations, since their usage in this study potentiated cell death.

5.3 RECOMMENDATIONS

In further studies, *in vivo* experiments in animal models can be conducted using the antioxidants (ascorbic acid and green tea Kombucha). Use of epithelial cells such as fibroblasts and keratinocytes in further studies will be necessary to confirm the protective effect of the exogenous antioxidants in these other wound-healing cells. Specific detection of hydrogen peroxide and hydroxyl radicals after mycolactone treatment will be more informative to confirm their involvement in mycolactone cytotoxicity.

5.4 LIMITATIONS

The volume of synthetic mycolactone A/B, which was a kind gift, was inadequate for the quantum of work in this research, thus the inability to perform more than one independent experiment in some of the generated results; to make them more reproducible.

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7.0 APPENDIX

I.

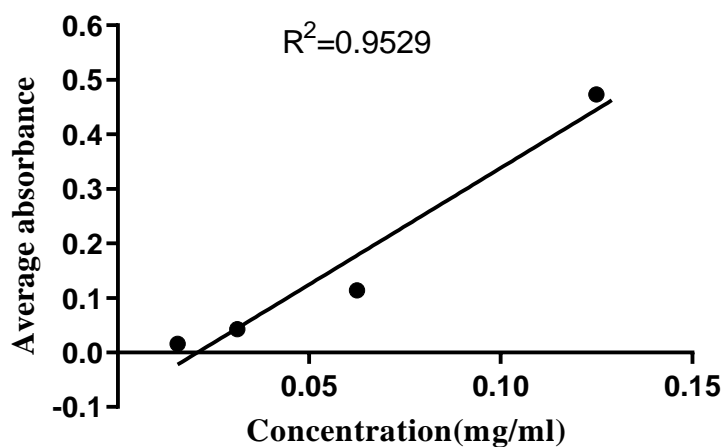


Fig 1: Calibration curve of gallic acid, serving as standard for total phenolic content determination in green and black tea Kombucha. The equation of the line of best fit

($y = 11.874x - 0.4148$) was used for calculating the phenolic content of both teas.

II.

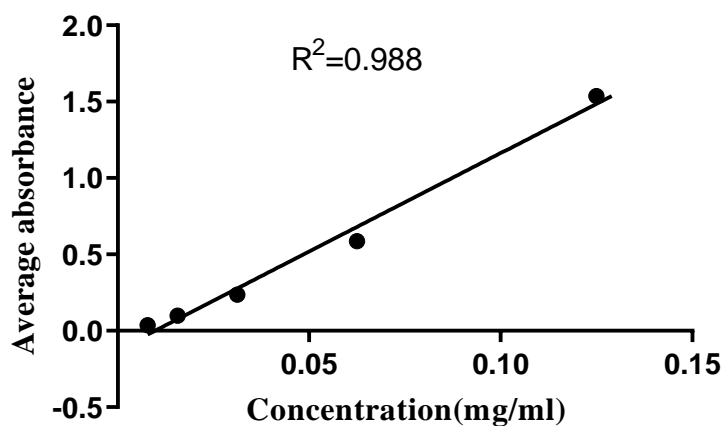


Fig 2: Calibration curve of quercetin, serving as standard for total flavonoid content determination in green and black tea Kombucha. The equation of the line of best fit was

($y = 8.9983x - 0.0833$) used for calculating the flavonoid content of both teas.

III.

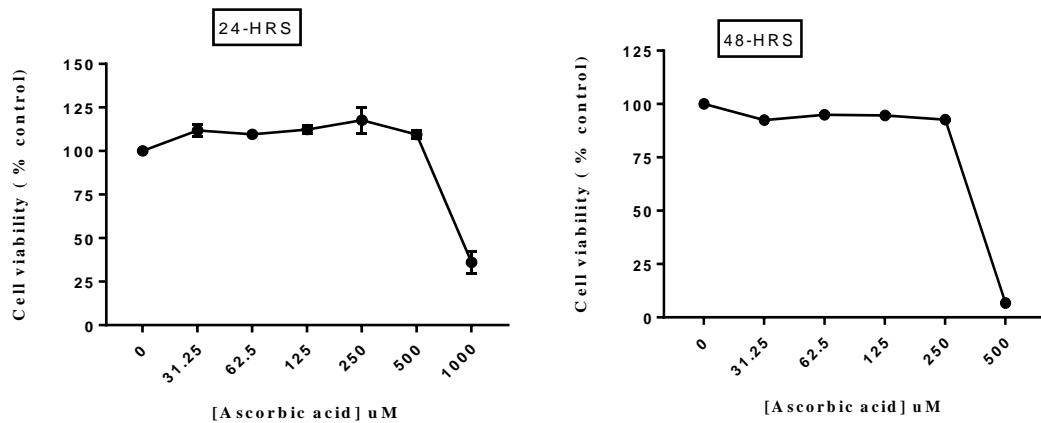


Fig 3: Cytotoxicity of ascorbic acid against RAW 264.7 macrophages after 24 and 48 hours. Cells were seeded overnight and treated with varying concentrations of the ascorbic acid for 24 to 48 hours. Cell viability was determined using Alamar blue.

IV.

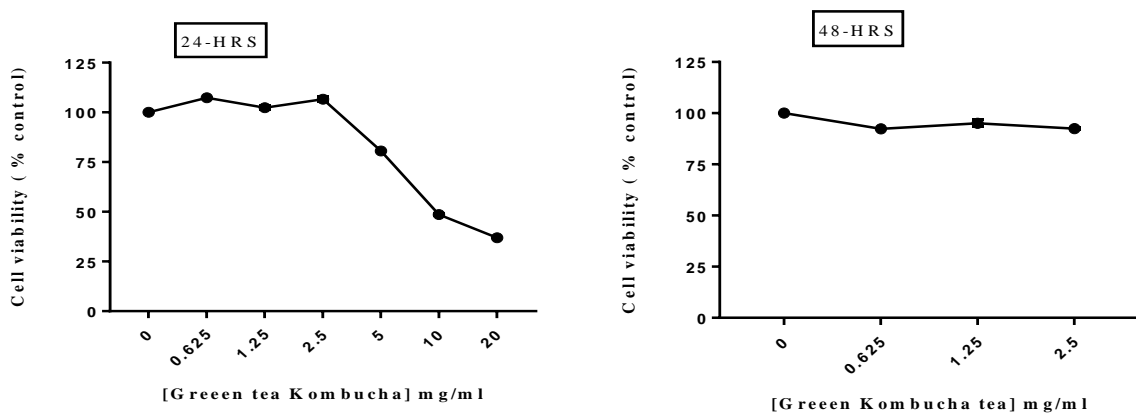


Fig 4: Cytotoxicity of green Kombucha tea against RAW 264.7 macrophages after 24 and 48 hours. Cells were seeded overnight and treated with varying concentrations of the green tea Kombucha for 24 to 48 hours. Cell viability was determined using Alamar blue.

V.

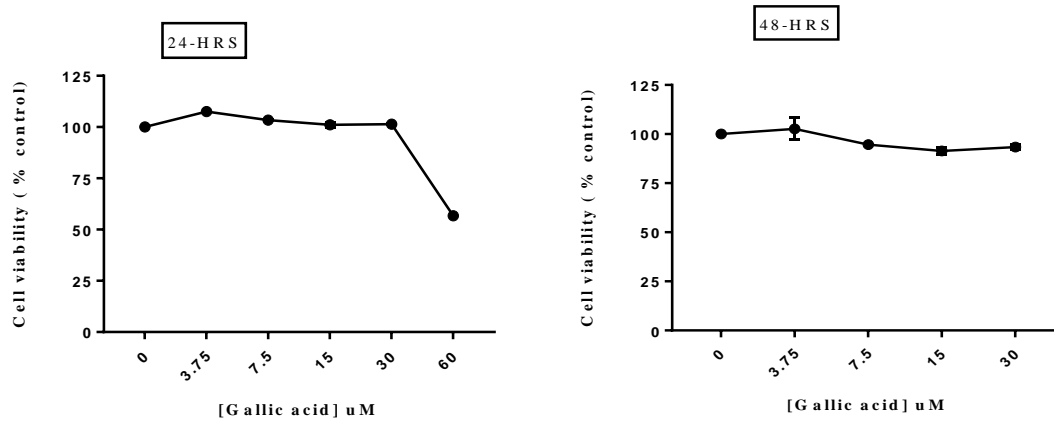


Fig 5: Cytotoxicity of gallic acid against RAW 264.7 macrophages after 24 and 48 hours.

Cells were seeded overnight and treated with varying concentrations of the gallic acid for 24 to 48 hours. Cell viability was determined using Alamar blue.