IN VITRO EVALUATION OF DIFFERENT TEMPERATURES ATTAINED DURING STEAM INHALATION WITH AND WITHOUT *EUCALYPTUS SALIGNA* FRESH LEAVES CRUDE EXTRACT ON SARS-COV-2 INFECTIVITY.

BY

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(210003414)

A RESEARCH STUDY SUBMITTED TO THE UNIVERSITY OF ZAMBIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE DEGREE IN ONE HEALTH LABORATORY DIAGNOSTIC SCIENCES

THE UNIVERSITY OF ZAMBIA

LUSAKA

DECLARATION

I, Chisanga Chipanta, declare that this Dissertation is my work and that all sources I have cited herein, have been indicated and acknowledged using complete references. I further declare that this Dissertation has not been previously submitted for a diploma, degree or any other qualifications at this or another university.

Signature……. **Date**……

CERTIFICATE OF APPROVAL

This dissertation of **Chisanga Chipanta** has been approved as fulfilling of the requirements or partial fulfillment of the requirements for the award of the Degree of Master of Science in One Health Laboratory Diagnostic Sciences by the University of Zambia.

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Examiner II

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Examiner III

Signature……... **Date**……

Board Chairperson

Signature……... **Date**……

ABSTRACT

Coronavirus disease of 2019 is an infectious disease caused by SARS-CoV-2. It is a highly transmissible emerging pathogen in humans. Primarily, infection happens when the spike glycoprotein on the surface of the virus binds to the host cell receptors resulting in a variate of asymptomatic and symptomatic infections. The disease's global case fatality, though dependent on the viral variant, ranged between 1.7% to 39% at the peak of the pandemic and there was no known medication to counter such effects. As a result, there was an urgent need for remedies to counter the disease. Among alternative remedies to conventional ones was steam inhalation, a notably typical home-setting remedy locally called *Ukufutikila* in Zambia. Studies have indicated that certain temperatures of steam when inhaled could inactivate SARS-CoV-2 although this information remains scanty. This study endeavored to determine the effects of temperatures attained during steam inhalation, a home remedy, both in the presence and absence of *Eucalyptus saligna* fresh leaves crude extract on the *in vitro* infectivity of SARS-CoV-2. Eight participants were recruited to each undergo steam inhalation for 20 minutes in which temperature variations were determined and ranged from 94.7°C to 54.4°C. These temperatures, based on the activity of Alkaline phosphatase (a surrogate glycoprotein), were then used to determine the *in vitro* infectivity of SARS-CoV-2. The SARS-CoV-2 material was inoculated into Vero T2 cells both in the presence and absence of *E. saligna* fresh leaves crude extract (13% in culture). Prior to inoculation, the SARS-CoV-2 material was heat treated following the determined temperature profile from steam inhalation. When SARS-CoV-2 material was heat treated or when inoculated in the presence of *E. saligna* fresh leaves crude extract, no observable cytopathic effects (0% of assays) were seen on the Vero T2 cells over a period of 72 hours. Withal, there was no significant virus growth ($p = 0.771$) estimated after exposure to the two treatments. The results observed in this study shed light on the possibility of steam inhalation being an efficient regimen in the management of COVID-19. In order to have more knowledge on the effects of steam inhalation temperatures on SARS-CoV-2 *in vitro* infectivity, this study recommends clinical trials involving COVID-19 asymptomatic patients and exposed frontline workers prior to onset of symptoms or immediately following exposure. Also, that further analyses on *E. saligna* fresh leaves crude extract be carried out to understand which phytochemicals are responsible for inhibitory effects on SARS-CoV-2 *in vitro* infectivity.

DEDICATION

I specially dedicate this piece of scientific literature to every researcher who is invested in seeking solutions and management options for the COVID-19 global pandemic that continues to ravage communities around the world to this day.

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LIST OF ACRONYMS

ACE2	Angiotensin Converting enzyme-2
ALP	Alkaline phosphatase
ANOVA	Analysis of Variance
ARDS	Acute Respiratory Distress Syndrome
AVE	Elution buffer
AVL	Viral lysis buffer
AW1	Wash buffer 1
AW2	Wash buffer 2
BSL-3	Biosafety level 3
CAM	Complementary and Alternative Medicines
CC_{50}	Cytotoxic Concentration
cm	centimeter
CoV	Coronaviruses
COVID-19	Corona Virus Disease of 2019
CT	Cytoplasmic tail
Ct	Cycle threshold
CTD	C-terminal domain
CTT	Cytotoxicity test
CPE	Cytopathic effect
DMEM	Dulbecco's Modified Eagle Medium
E	Envelope protein
ERES	Excellence in Research Ethics and Science
FBS	Fetal Bovine Serum
FP	Fusion peptide
g	grams
H1N1	Swine flu
HE	Hemagglutinin-esterase

HR	Heptad repeat
IRB	Institutional Review Board
l	litre
LMICs	Low/Middle-Income Countries
m	meter
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
mg	milligrams
ml	millilitre
mm	millimeter
N	Nucleo-capsid protein
NHRA	National Health Research Authority
NTD	N-terminal domain
ORFs	Open reading frames
O_2	Oxygen
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PI	Principle Investigator
PIS	Participant Information Sheet
pNPP	4-nitrophenyl phosphate
RBD	Receptor-Binding Domain
RBM	Receptor-Binding Motif
RDT	Rapid Diagnostic Test
RNA	Ribonucleic Acid
RT-qPCR	Reverse Transcriptase Quantitative Polymerase Chain Reaction
S	Spike glycoprotein
S1	Subdomain 1
S2	Subdomain 2
SARS	Severe Acute Respiratory Syndrome

SARS-CoV	Severe Acute Respiratory Syndrome Coronavirus
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus-2
SEM	Standard Error of Mean
TB	Tuberculosis
TM	Trans-membrane
TMP	Traditional Medicine Practitioners
UNZA	University of Zambia
URS	Upper Respiratory System
VOC	Variants of Concern
VOI	Variants of Interest
VTM	Virus transport media
WHO	World Health Organization
°C	Degree Celsius
µl	Microlitre

CHAPTER ONE

1.0 INTRODUCTION

1.1 COVID-19

Corona Virus Disease of 2019 (COVID-19) is a viral disease caused by Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2). It was first reported in Wuhan, China in December 2019 after an initial cluster of pneumonia-like cases. It is a positive sense single-strand Ribonucleic Acid (RNA) virus that primarily affects respiratory tract epithelial cells. It is a highly transmissible emerging pathogen in humans and individuals infected by the virus experience inflammations of the mucosal membrane that damages alveoli and result in pneumonia (Rafiqul Islam *et al.*, 2021). The mode of transmission is through respiratory droplets following close contact with an infected individual (Manon *et al.*, 2021).

According to the WHO (World Health Organization) COVID-19 Dashboard, there has already been a global cumulative total of 769,774,646 cases, 6,948,751 deaths with more expected (WHO, 2023). As of August 16, 2023, COVID-19 statistics for Zambia's daily updates indicated a cumulative total of over 349,287 positive cases with a 4,069 death toll (Ministry of Health, 2023).

1.2 Pathogenesis and symptoms

After being infected by the virus, individuals exhibit three stages of viral progression. Initially, the spike glycoprotein (S) binds to the Angiotensin Converting Enzyme – 2 (ACE2) on human cell receptors in the nasal epithelium and begins replicating (Chowdhury *et al.*, 2022). This is known as stage 1 of the infection. Here, patients are usually asymptomatic and show little to no symptoms. Stage 2 involves invasion of the Upper Respiratory System (URS) where virus propagation and migration advances. Patients begin showing mild or significant symptoms like fever, running nose, myalgia and dry cough among others. By stage 3, the infection symptoms become more severe and individuals experience hypoxia with ground glass pulmonary infiltrates. This progresses to a critical phase known as Acute Respiratory Distress Syndrome (ARDS). The ARDS may include shock and coagulation defects. About 20% of COVID-19 patients progress to the third stage

(Sarker, 2021). The zoonotic transmission of COVID-19 stokes a huge human health threat requiring urgent public health initiatives that may help contain the pandemic (Zhang *et al.*, 2021).

Patients with COVID-19 may experience fever, tiredness and dry cough as symptoms accompanied by pain in joints, nasal congestion, running nose, sore throats and diarrhoea (Orisakwe *et al.*, 2020). Studies assessing the diagnostic accuracy of the signs and symptoms of COVID-19 have shown that cough or fever are useful indicators for isolating suspected patients for further testing (Struyf *et al.*, 2021). Moreover, additional research has revealed that the most common symptoms among COVID-19 patients included fever, cough, fatigue and dyspnea. Thus, from the main COVID-19 symptoms, cough, fever, fatigue and dyspnea play a prominent role in the early detection of the disease (Alimohamadi *et al.*, 2020).

1.3 Control of COVID-19

Studies indicate that modalities in developing therapeutic regimens against SARS-CoV-2 may take a while to be realized. Therefore, the focus is skewed toward exploring alternative medicines that will likely alleviate COVID-19 symptoms. Largely, this has been the practice in African societies where people resort to traditional/home remedies (Nur *et al.*, 2020).

The management of COVID-19 can often lie outside the means of an average person. This is because it requires proper medical facilities which are usually unavailable in remote or rural parts of low and middle-income countries (LMICs) (Anam *et al.*, 2021). Particularly in Zambia, the majority of confirmed cases are under community management as ≤1% are managed at health facilities. Patients on community management were advised to use vitamin supplements, staying hydrated, exercising, eating a balanced diet and steaming up (*Ukufutikila*). While those at health facilities were managed using various treatment regiments like heparin, remdesivir, dexamethasone, various antibiotics or plasmapheresis for those under critical care (Ministry of Health, 2023). Most developing countries continue to rely heavily on traditional medicines as major therapeutic options due to the high expense and irregular supply of conventional medicines. Therefore, to harness its potential in the health system, the WHO has since began shaping guidelines and policy recommendations

on traditional/complementary medicines for countries. This is because for millions, it is the first stop for healthcare and well-being (WHO, 2023). With evidence of its effect on influenza strains like the Swine flu (H1N1), the Chinese government has also supported the use of traditional medicines in the management of COVID-19 (Rafiqul Islam *et al.*, 2021).

1.3.1 Prevention

Currently, the main focus of COVID-19 prevention and control is on early detection, diagnosis, isolation and treatment (Wu *et al.*, 2020). Hygienic practices like frequent hand washing with soap and hand sanitizing have been emphasized in COVID-19 prevention. Likewise, coughing and sneezing into the elbow and wearing facemasks, are among the measures that are continuously being promoted in COVID-19 prevention. However, the safest mode of COVID-19 prevention has been optimizing individual risk management and reducing disease severity in infected individuals (Ağagündüz *et al.*, 2021). Therefore, the first line of disease management has always been subjecting suspected patients to periodical quarantines (Kaye *et al.*, 2021).

Additionally, following the discovery of various forms of vaccines (Creech *et al.*, 2021), there has been an advocate for people to get vaccinated against COVID-19. The emergence of several new strains of SARS-CoV-2 for most of 2021, propelled more effort into the development of newer vaccines (Hossain *et al.*, 2021).

1.3.2 Vaccines

Vaccination has played a pivotal role as an intervention strategy in the ongoing COVID-19 pandemic. As of August 15, 2023, a total number of 13,498,570,620 had been administered (WHO, 2023). Essentially, Zambia has achieved a vaccination rate of ~ 80% of the targeted population (Ministry of Health, 2023).

Available vaccines are of different types and may cover protein-based vaccines, DNA, RNA and Viral vector-based vaccines. Depending on the type of vaccine, the mechanism used to evoke immune response differs (Table 1.1). Some examples of the available vaccines include mRNA-1273 vaccine by Moderna, AZD1222 by AstraZeneca and University of Oxford, BNT162 vaccine by Pfizer and BioNTech as well as COVID-19 vaccine by Sinopharm and the Wuhan Institute of Virology, China among others (Kashte

et al., 2021). Fundamentally, current vaccines are based on destabilizing the S protein of the SARS-CoV-2 and disrupting or weakening the Receptor-Binding Domain (RBD) interactions. Furthermore, research is being conducted to investigate vaccines designed to target the RBD, the Subdomain 1 (S1) and the Subdomain 2 (S2) subunits of the SARS-CoV-2 glycoprotein for protective effects against the virus (Yan *et al.*, 2021).

Table 1.1 Different types of vaccines approved for use by WHO.

Type	**Example**	**Mechanism**
Inactivated/weakened virus vaccines	Sinovac-Coronavac, Sinopharm, Bharat and Valneva	Use a form of the virus that has been inactivated or weakened so that it does not cause disease but still generates immune response.
Protein-based vaccines	Novavax	Use harmless fragments of proteins that mimic the COVID-19 virus to safely generate immune response.
Viral vector vaccines	AstraZeneca/Oxford, Janssen and CanSino	Use a safe virus that cannot cause disease but serves as a platform to produce coronavirus proteins to generate immune response.
RNA and DNA vaccines	Pfizer/ BioNTech, Moderna	Use genetically engineered RNA or DNA to generate a protein that safely prompts immune response.

Adapted from: World Health Organization, 2023

1.3.2.1 Vaccines acceptability

Particularly in 2021, the WHO reported significantly low vaccine coverage (WHO, 2021). Of growing concern was the reluctance of people to take recommended and safe vaccines, a trend known as vaccine hesitancy. This had unfortunately been among the attributed factors of low vaccine coverage.

In LMICs there had been visible hesitancies attributed to low literacy levels especially that communities have exhibited fear of vaccine side effects and a general lack of confidence in vaccine effectiveness (Bono *et al.*, 2021). Other reasons for vaccine hesitancies included the association with age, gender, income, residency, occupation and race/ethnicity as the general public perceived potential vaccine harms. More males than females expressed concern for vaccines as they perceived health related risks or potential harm after vaccination. However, overall the younger individuals expressed more collective responsibility and confidence to get vaccines. In most African communities, people were seen to be less knowledgeable about vaccines hence the high prevalence in vaccine hesitancy (Wake, 2021). On the other hand, people who were more educated, or had higher income were more knowledgeable about vaccines and where seen to exhibit low vaccine hesitancy (Bono *et al.*, 2021). Furthermore, mistrust of the healthcare system and the use of social media for COVID-19 vaccine-related information were major barriers influencing vaccine uptake (Wake, 2021). However, the concerns about the side effects were the most projected (Machingaidze and Wiysonge, 2021).

Studies have recommended that trust and confidence in the COVID-19 vaccines would be crucial to their success (Knoll and Wonodi, 2021). Nevertheless, there remains an earnest need for effective therapeutic alternatives in the prospect of efficient containment of the pandemic (Xiaohui *et al.*, 2021).

1.3.3 Conventional treatment - repurposed drugs and new drugs

The treatment of COVID-19 has focused on the repurposed use of drugs (Agrahari *et al.*, 2021; Riva *et al.*, 2020; McKee *et al.*, 2020). Chloroquine and hydroxychloroquine used in malaria and amoeba treatments have been seen to be effective in blocking the RBD-ACE2 interactions and reducing the replication rate of SARS-CoV-2, respectively (Shagufta and

Ahmad, 2021). Favipiravir which is antiviral against influenza has also been observed to have an *in vitro* reduction of the SARS-CoV-2 infectivity rate (Joshi *et al.*, 2021).

Several drugs have been investigated for both efficacy and safety in COVID-19 treatment with clinical trials conducted on most drugs being used in COVID19 therapy (Tarighi *et al.*, 2021). Previous studies have determined that there is an immense need to understand the lifecycle of SARS-CoV-2 to identify drug targets in coming up with effective therapeutics (Gil *et al.*, 2020).

Some classes of drugs have been utilized in the early treatment of COVID-19 on the basis of its pathology (Ceramella *et al.*, 2022). These include antiviral drugs, antimalarial drugs and antibiotics among others (Table 1.2).

Table 1.2. Some classes of drugs being utilized in the treatment of COVID-19 based on its pathological effects.

Class	**Pathological Effect**	**Example**
Antiviral drugs	Prevention of virus entry, suppression of replication and assembly steps.	Remdesivir, Favipiravir,
Antimalarial drugs	Reduces viral load	Chloroquine and Hydroxychloroquine Chloroquine
Antibiotics	Reduces viral load	Azithromycin
Interleukine Inhibitors	Reduces hyper-inflammatory markers	Anakinra
Janus Kinase Inhibitors	Reduces levels of inflammatory markers and improves oxygenation	Baricitinib
Corticosteroids	Reduces hyper-inflammation in severe and critical patients	Dexamethasone
Anticoagulants	Management of inflammatory and coagulative in advanced COVID-19 phase.	Low molecular weight heparin
Non-steroids Anti-inflammatory drugs	Pain relief and reduction of inflammations	Ibuprofen

Adapted from: Ceramella *et al.*, 2022

Fundamentally, here has been a continued search for regimens and credible solutions with traditional/home remedies being reported to alleviate most symptoms (Orisakwe *et al.*, 2020). Several herbal medicine candidates have been identified as a possible complementary treatment for COVID-19 with reports of significant use of medicinal plants for COVID-19 in different parts of the world (Nugraha *et al.*, 2020; Orisakwe, Orish and Nwanaforo, 2020; Villena-Tejada *et al.*, 2021).

1.3.4 Alternative remedies to COVID-19 treatment

According to research, most members of the public and patients were seen to rely on self-care practices in trying to relieve symptoms of COVID-19 or as a prevention measure. This utilization of practices not fully integrated into the healthcare system is referred to as complementary and alternative medicines (CAM) (Paudyal *et al.*, 2022). Some of the practices in CAM include use of herbs in the treatment of respiratory infections (Gbadamosi, 2020). Nonetheless, with the COVID-19 pandemic, herbal remedies were among the most commonly self-prescribed medications (Malik *et al.* 2020). In Africa, there is an estimation of 80% of the population using home/traditional remedies for the treatment of COVID-19. However, the practice of using CAM was seen to be common across the globe during the COVID-19 pandemic (Paudyal *et al.*, 2022).

Furthermore, it has been observed that steam inhalation, an inexpensive, self-manageable process, could reduce COVID-19 symptoms in patients. Steam inhalation is a notably typical home-setting approach. It has been practiced to heal infections associated with the respiratory system like the Rhinovirus-mediated common cold and influenza among others (Chowdhury *et al.*, 2022). The effects of steam inhalation on other respiratory infections could give indirect evidence regarding its potential to deactivate SARS-CoV-2 (Swain and Sahu, 2021). Therefore, conducting experiments with a heat stable protein like Alkaline phosphatase (ALP) (Liu *et al.*, 2013) would give a close approximation of this deactivation. For this reason, the ALP would act as the chosen surrogate protein of SARS-CoV-2 spike membrane protein in this study.

Moreover, studies have also shown that traditional herbal remedies may help enhance a person's immunity and alleviate symptoms of COVID-19 (Nur *et al.*, 2020). One of the

most used herbal remedy for various medicinal purposes including influenza are the leaves of the *Eucalyptus* spp. (Döll-Boscardin *et al.*, 2012).

Studies have reported use of antibodies that target the S1 subunit of the spike glycoprotein as a first line of treatment. This is mainly because the spike glycoprotein acts as the main factor in adhesion and virulence in host cells. Being the prime interacting protein with host cell targets, it is seen to potentially assist the development of future therapeutics against SARS-CoV-2 (Vankadari and Wilce, 2020).

1.4 COVID-19 impact

According to research, COVID-19 has caused global stress and public health emergencies including economic crises and millions of infections all over the world (Mittal *et al.*, 2020). The COVID-19 pandemic has particularly seen a huge loss of life (Bundervoet *et al.*, 2022). As of August 16, 2023, the WHO reports a cumulative total of over 6.9 million worldwide deaths (WHO, 2023). Zambia singly recorded 4,069 deaths by August 16, 2023 (Ministry of Health 2023).

Zambia, like most LMICs, has been impacted by paramount pressure on its health systems. With significant Oxygen (O_2) shortages causing preventable deaths, hospitals, patients and families have been subjected to desperate situations (Usher, 2021). What is worse, the limited infrastructure and low vaccine coverage still cannot attract enough support from the political reality of richer countries (Miguel and Mobarak, 2021). The ubiquitous and life-threatening infectious nature of COVID-19 along with the consistent global and national updates, have caused a lot of anxiety and fear (Roelen *et al.*, 2020).

Sound reactions from Governments the world over, resulted in the formulation of restrictions and bans on most social programs (Dupas *et al.*, 2022). As a result of these lockdowns, home confinements have particularly had a negative bearing on the mental health of children and youths. Usually, these have been forced to do less work and withdraw from their peers. However, it has still been difficult to detect mood and anxiety disorders which are among the commonest disorders in young adults and children (Courtney *et al.*, 2020).

Developed as well as LMICs have been severely affected by economic contraction. Even

though the effects of job losses in regions like Africa where economic activities largely depend on personal interactions have been more adverse. About a billion students have faced interruptions in their school schedules worldwide and this poses magnanimous long-term effects. Another worsening aspect of livelihoods in vulnerable regions of the world has been food security, especially following indefinite lockdowns and job pauses (Bundervoet *et al.*, 2022). The COVID-19 pandemic had considerable impact on agricultural households (Nolte *et al.*, 2022). The survey data from Burkina Faso, Chad, Ethiopia, Malawi, Mali, Nigeria, Uganda and Zambia, revealed that Zambia had the highest rate of difficulty to access agricultural activities and markets (at 20%). This was attributed to income decreases and increases in the pricing of commodities. There is a need to have an active concern over policy responses to the pandemic as they have much more impact on the more vulnerable populations (Dupas *et al.*, 2022).

1.5 Statement of the problem

While COVID-19 preventive measures have been endorsed by various governments, there seem to be considerably low adherence levels (Carcelen *et al.*, 2022). Limited access to healthcare especially in LMICs with limited resources (Ali *et al.*, 2021) substantiates a need to validate alternative remedies currently in use. Steam inhalation along with or without herbal extracts has been used as an alternative home remedy even before the onset of the COVID-19 pandemic. Yet, its effectiveness remains to be fully determined experimentally to justify its use in the alleviation of COVID-19 symptoms. This may possibly result in curbing the spread of the SARS-CoV-2 virus while utilizing cheap and readily available practices in local communities. Understanding the effects of different temperatures attained during steam inhalation with and without *Eucalyptus saligna* fresh leaves crude extract on the SARS-CoV-2 could be a critical step in improving the general response to the pandemic in terms of prevention or management.

1.6 Rationale of study

This study endeavored to establish information that would possibly confirm an alternative to COVID-19 management and presumably reduce the infectivity rate among populations, especially in low/middle-income countries like Zambia with limited medical facilities.

1.7 Hypothesis

What are the possible effects of temperatures attained during steam inhalation both in the presence and absence of *E. saligna* fresh leaves crude extract on the *in vitro* infectivity of SARS-CoV-2?

1.8 Study objectives

1.8.1 Main objective

To determine the effects of temperatures attained during steam inhalation as a home remedy both in the presence and absence of *E. saligna* fresh leaves crude extract on *in vitro* infectivity of SARS-CoV- 2.

1.8.2 Specific objectives

1. To determine temperature variations attained during steam inhalation.
2. To identify specific temperatures from the determined temperature profile of steam inhalation that reversibly or irreversibly affect ALP *in vitro* activity.
3. To determine *in vitro* infectivity of SARS-CoV-2 exposed to the identified temperatures (in ii above), in the presence and absence of *E. saligna* fresh leaves crude extract, of Vero T2 cells.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Coronaviruses

Generally, Coronaviruses (CoV) are classified into four genera which include α-/β-/γ-/δ-CoV with α- and β-CoV being considered infectious agents for mammals. Whereas γ- and δ-CoV are considered pathogens for birds (Agrahari *et al.*, 2021).

In 2003, a Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) emerged and caused a major human epidemic (Qing and Gallagher, 2020). Its first occurrence was in the Guangdong Province of China in November 2002. By 2005, evidence strongly indicated that the 2003 Severe Acute Respiratory Syndrome (SARS) epidemic and the 2004 mild SARS cases were harboured by the masked palm civets. Additionally, bats were found to be natural reservoirs of SARS-CoV-like viruses with genetic diversity of such coronaviruses (Shi and Hu, 2008).

A subsequent coronavirus emerged in 2012 and was called the Middle East Respiratory Syndrome Coronavirus (MERS-CoV). It initially originated from Bisha in the Kingdom of Saudi Arabia and then spread to Europe and parts of Africa. Since its discovery, MERS-CoV has now been found in bats and dromedary camels as reservoir animals (Mackay and Arden, 2015).

2.2 SARS-CoV-2

Essentially, SARS-CoV-2 belongs to the subfamily Orthocoronavirinae of the family Coronaviridae whose most members are zoonotic in nature. It has been studied to have a spherical external spike protein that displays a characteristic crown under the electron microscope (Dawood, 2020).

As the virus spreads between people, its genome normally undergoes mutations. It hence becomes significantly different from the original genetic material. These significantly different virus strains are known as variants. A variant becomes a Variant of Interest (VOI) when mutations impact its spread, severity, vaccine performance, diagnostic tools therapeutic medicines and leads to replacement of circulating variants. On the other hand,

if a VOI begins to spread more easily, it is defined as a Variant of Concern (VOC) (Dol *et al.*, 2022). Scientists can map these changes and globally track the emergence of such newer strains. Thus, for easy reference and media/public discussion, the WHO necessitated a naming system using the Greek alphabet in May 2021. However, this necessitated naming system still does not replace the scientific name classification (WHO, 2021). In this regard, the WHO specifically classifies VOCs uniquely assigned by Greek labels (Table 2.1). Currently, the most dominant variant is the Delta variant although the Omicron variant has caused a detrimental shift in the epidemiology of the COVID-19 pandemic (WHO, 2021).

Table 2.1. Currently and previously circulating COVID-19 VOC. *Includes all descendent lineages.

WHO label	**Pango lineage**	**Designation**	**Date designated**	**Country first detected**
Omicron*	B.1.1.529	VOC	November 2021	Multiple countries
Alpha	B.1.1.7	VOC	December 2020	United Kingdom
Beta	B.1.351	VOC	December 2020	South Africa
Gamma	P.1	VOC	December 2020	Brazil
Delta	B.1.617.2	VOC	June 2021	India
Epsilon	B.1.427/B.1.429	VOC	March 2021	United States of America

Adapted from: Centers for Disease Control and Prevention, 2023

2.3 Structure of SARS-CoV-2

SARS-CoV-2 is a single-stranded RNA-enveloped virus. Its genome size is approximately 29.9 kb. The genome encodes 9860 amino acids resulting in four structural proteins as well as sixteen non-structural proteins (Wang *et al.*, 2020). The most prominent structural features of SARS-CoV-2 are the Spike glycoprotein (S), Membrane protein (M), Nucleo-capsid protein and Envelope protein (E) (Figure 2.1). The M protein is the most abundant protein of SARS-CoV-2. It is a triple helix bundle having a sugar transporter-like structure (Sunil, 2020). The M protein defines the shape of the viral envelope together with the E

protein. It is also regarded as the central organizer of the CoV assembly as it interacts with all other major structural proteins (Schoeman and Fielding, 2020). Present on its cell membrane surface, are the spike glycoproteins (Huang *et al.*, 2020). A large number of glycosylated S proteins cover the surface of SARS-CoV-2. The presence of the Hemagglutinin-esterase (HE) enhances the ability of the virus to bind to the host cell, especially in novel CoVs (Felsenstein *et al.*, 2020).

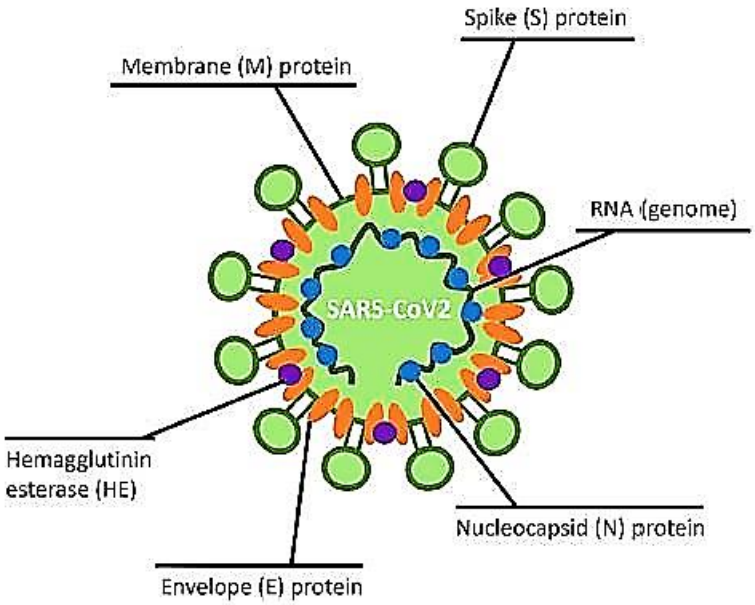

Figure 2.1. Structure of SARS-CoV-2. The S protein facilitates binding to the trans-membrane ACE2 host receptor; the E protein together with the M protein forms the viral envelope and determines its shape; the HE protein may resemble another cell entry mechanism of novel CoVs; the N protein inbound to the RNA genome of the virus to form the nucleocapsid (Source: Felsenstein et al., 2020).

2.3.1 Spike glycoprotein

The SARS-CoV-2 spike glycoprotein is a 1273 amino acid polyprotein precursor and is central in viral infection and pathogenicity. Structurally, it is made up of two subunits. The S1 subunit acts as the receptor binding domain while the S2 subunit acts as the stalk, providing the spike structure (Figure 2.2). These act as the vital pathogenic features of the virus (Agrahari *et al.*, 2021).

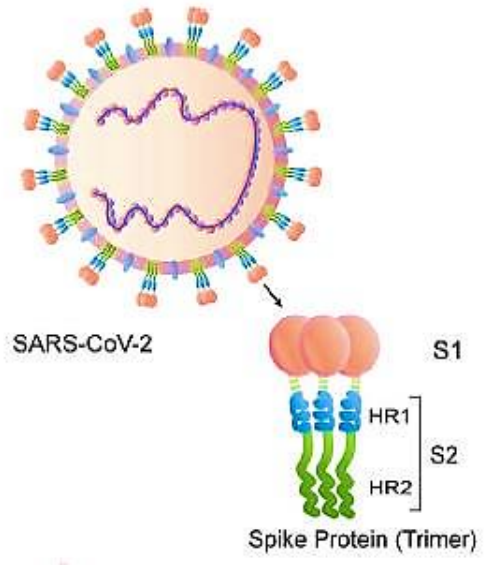

Figure 2.2. The schematic structure of the S protein. (Source: Y. Huang et al., 2020).

The surface S1 subunit is composed of about 672 amino acids with four domains; the N-terminal domain (NTD), C-terminal domain (CTD); and two subdomains, subdomain 1 (S1) and subdomain 2 (S2) (Figure 2.3). However, the trans-membrane S2 subunit composed of about 588 amino acids is made up of the N-terminal hydrophobic fusion peptide (FP), 2 heptad repeats (HR_1 and HR_2), trans-membrane (TM) and the cytoplasmic tail (CT) which are arranged as FP-HR_1-HR_2-TM-CT (Duan *et al.*, 2020).

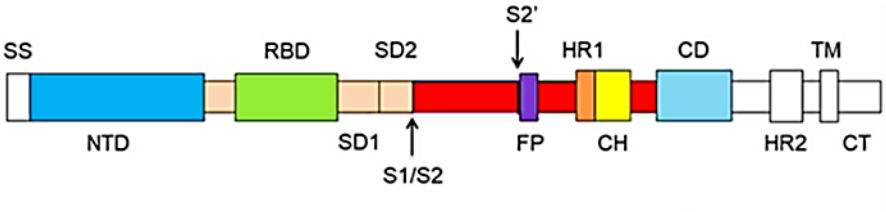

Figure 2.3. Schematic diagram of SARS-CoV-2 spike protein primary structure. Different domains are shown in different colors. SS, single sequence; NTD; RBD; S_1; S_2; S1/S2, S1/S2 protease cleavage site; S2', S2' protease cleavage site; FP; HR1; CH, central helix; CD, connector domain; HR2; TM; CT (Source: Wang et al., 2020).

2.3.2 Entry of SARS-CoV-2 into host cells

The spike glycoprotein present on its cell membrane surface is responsible for the initial binding to host cell receptors (Figure 2.4) and results in infection (Huang *et al.*, 2020). The RBD is primarily responsible for binding the ACE2 on host cell surfaces. This is because of the glycosylation sites found in the S1 subunit which facilitate covalent bond formation.

Furthermore, the heavily glycosylated N- and O- ends complement host cell recognition and viral entry including immunological systems interactions and potential future diagnostic developments (Antonopoulos *et al.*, 2020).

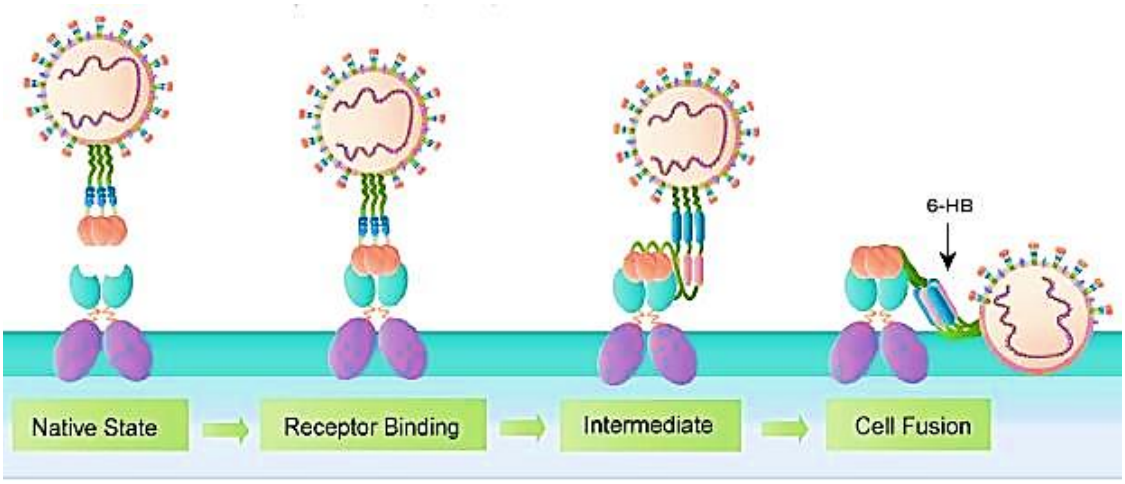

Figure 2.4. A stepwise SARS-CoV-2 mechanism of entry in host cells. The S protein mediates the binding and virus–cell fusion process when it interacts with the ACE2 receptors on host cells (Source: Huang et al., 2020).

The membrane protein of the SARS-CoV-2 plays a critical role in the infection and life cycle of the virus facilitating receptor recognition and attachment to host cells as well as the entry (Huang *et al.*, 2020). The successful entry of the viral particle into the host cell to cause an infection depends on a functional SARS-CoV-2 membrane spike protein which is a glycoprotein (V'kovski *et al.*, 2021). Therefore, it has been identified as a possible target in the disruption of infections (Huang *et al.*, 2020). Viral fusion happens in such a way that the spike glycoprotein fluctuates from its closed conformation to its open conformation which mediates a hydrophobic bond formation between the RBD and the ACE2 (McCallum *et al.*, 2020). In providing an overview of drug targets while searching for therapeutic options for SARS-CoV-2, research has observed additional polar interactions (Gil *et al.*, 2020). This consisted of a hydrogen-bond interaction between Q439 of S-RBM (Receptor-Binding Motif) and E35 of ACE2 and one salt bridge between K417 of S-RBM and D30 of ACE2 in the central part of the S-RBM−ACE2 surface (Figure 2.5). This interaction was regarded as a clear effect of the different electrostatic profiles. The weak secondary bonds (hydrogen bond, hydrophobic interaction and electrostatic interaction) facilitating the RBD and ACE2 receptors fusion, may form a basis for disruption of this interaction through their

denaturation through steam inhalation. The disruption of these forces would be accompanied by the disturbance of the three-dimensional structure of the spike protein. This may result in a botched SARS-CoV-2 infection of host cells.

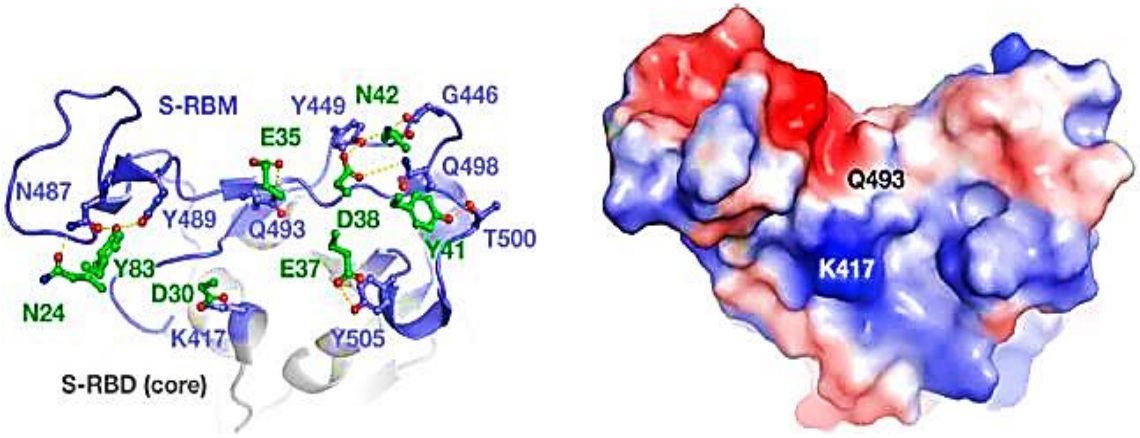

Figure 2.5. S-RBM (in violet) of S-RBD and ACE2 (in green) polar interactions for SARS-CoV-2. Additional polar contacts (Q493-E35 and K417-D30) are visible in the central region of the contact surface (Source: Gil et al., 2020).

2.3.3 Life cycle of SARS-CoV-2

After successful virus entry, the lifecycle of SARS-CoV-2 then proceeds to replication, assembly and release (Figure 2.6). Replication happens when the infecting RNA acts as messenger RNA and is translated to produce viral replicative enzymes. These then generate new RNA genomes and messenger RNAs that will synthesize components needed to assemble new viral particles in the cytoplasm. Subsequently, virus assembly of nucleocapsids follows right after the translation which bud into the lumen of the Endoplasmic Reticulum (ER)-Golgi compartment. Finally, release of virions then proceeds via exocytosis where they acquire their cell membranes from the infected cell (Zatla *et al.*, 2021).

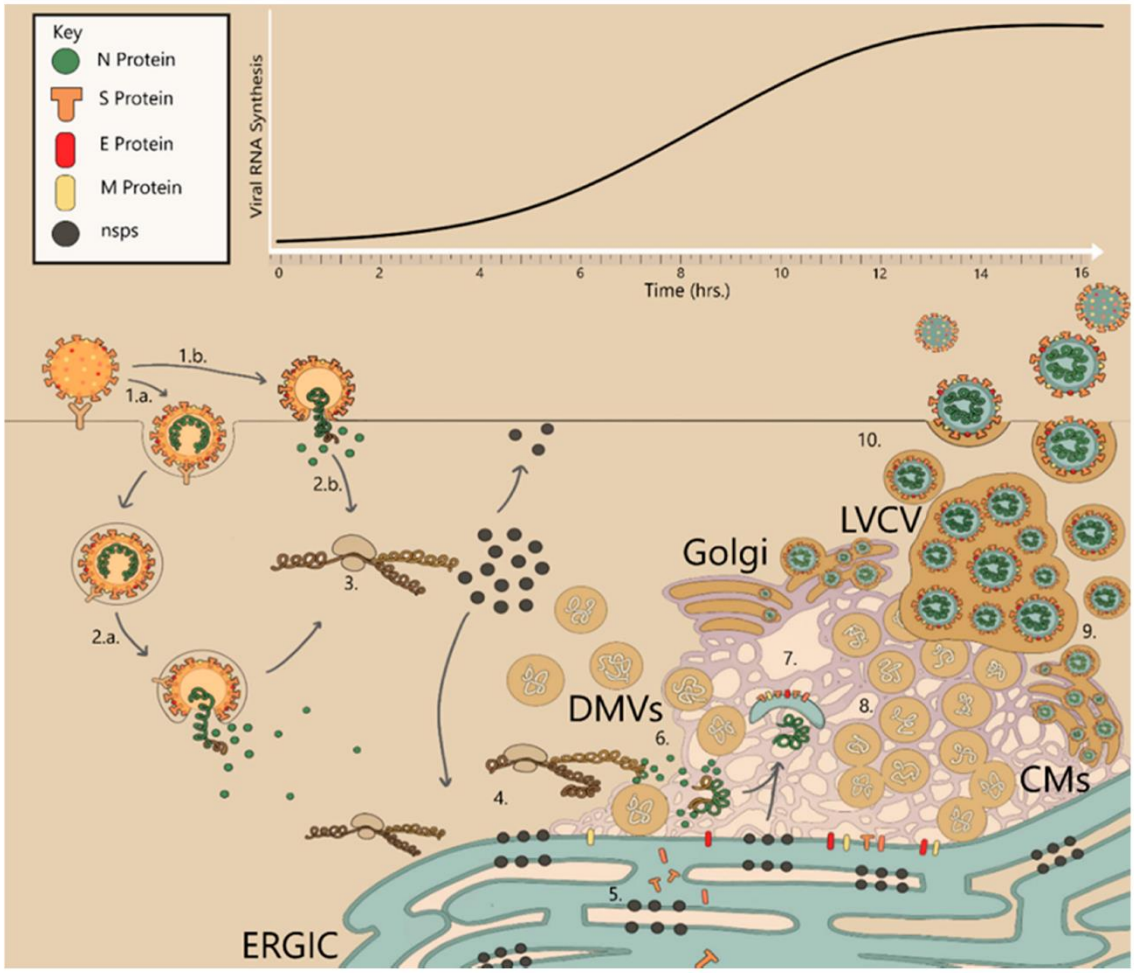

Figure 2.6. Showing the lifecycle of SAR-CoV-2 (Source: Wong and Saier, 2021).

2.3.4 Alkaline phosphatase

SARS-CoV-2 is categorized as a biosafety level 3 (BSL-3) agent and this impedes most research into its biology (Stelzer-Braid *et al.*, 2020). Hence use of ALP as a glycoprotein to mimic the viral membrane spike protein's response to heat would give a close approximation of its behaviour (Fujinami and Peterson, 2007) at different temperature profiles attainable during steam inhalation. Besides, the use of ALP to obtain baseline information of effect of heat on glycoprotein activity would abate the cost of conducting this study. This baseline data would then act as a guide on how to proceed with the thermal treatment experiments of SARS-CoV-2 infectious material in this study.

Furthermore, ALP is a ubiquitous membrane-bound glycoprotein that fundamentally catalyzes the hydrolysis of phosphate monoesters. It exists as four isoenzymes; intestinal

ALP, Placental ALP, Germ cell ALP, tissue non-specific or Liver/Bone/Kidney ALP (Sharma *et al.*, 2014). The ALP also takes the form of a dimer consisting of two identical subunits and usually has been observed to have a significantly high thermal resistance. However, conformational changes like the sequential unfolding of the protein changes are reported to be seen with the increase in temperature. These conformational changes resulted in inactivation of ALP (Dumitrascu *et al.*, 2015). This is as a result of the thermal disruption of its conserved hydrophobic residues accompanied by ionic, acidic or basic residues (Vimalraj, 2020). It is worth noting that some of these bonds, form the basis of SARS-CoV-2 RBD and ACE2 receptors interaction (section 2.3.2). Hence, disruption of these bonds through heat treatment would compromise the integrity of protein structure. Furthermore, heat inactivation experiments have been conducted on ALP in which temperatures similar to those used in the steam inhalation were employed (Moss and Whitby, 1975). Studies have shown that ALP is a highly attractive fusion partner due to the fact that it forms homodimers, thus, is a heat stable protein (Liu *et al.*, 2013).

2.4 Alternative remedies

2.4.1 Use of antibodies

Notably, all proteins encoded by SARS-CoV-2 genome are antivirus drug targets but the antibodies usually target the spike glycoprotein to block viral entry. Some therapeutics that have been developed in the use of antibodies include anti-virus monoclonal antibody therapeutics. For example, bamlanivimab which appeared to accelerate viral load decline. On the other hand, other studies have delved into anti-host monoclonal antibody therapeutics. For example, tocilizumab, a monoclonal antibody targeting IL-6R used in critical COVID-19 patients treatment (Ning *et al.*, 2021). More examples of antibodies that have been developed are shown in Table 2.2 below.

Table 2.2 Showing Anti-virus monoclonal antibodies (A) and Anti-host monoclonal antibodies (B) for COVID-19 treatment.

Name	**Target**	**Status**	**Developer**
A.			
Bamlanivimab	Spike protein	Emergency use authorization (USA)	Eli Lilly
Sotrovimab	Spike protein	Phase 3	Vir biotechnology/GSK
Regdanvimab	Spike protein	Phase 3	Celltrion
TY027	Spike protein	Phase 3	Tychan
B.			
Levilimab	IL-6R	Approved (Russia)	BIOCAD
Itolizumab	CD6	Emergency use authorization (India)	Biocon
Tocilizumab	IL-6R	Phase 4	Roche
Ravulizumab-cwvz	C5	Phase 4	Alexion Pharmaceuticals
Sarilumab	IL-6R	Phase 4	Regeneron
Olokizumab	IL-6	Phase 3	R-Pharm JSC/Cromos Pharma

Adapted from: Ning *et al.*, 2021

2.4.2 Herbal remedies

Although prospects have indicated better healthcare with reduced economic costs on drugs, scientific knowledge attesting the safety and efficacy of herbal remedies has been limited. Withal, several respiratory conditions have made use of steam inhalation as an adjunct and treatment in traditional home remedies (Marvin *et al.*, 2020). Steam inhalation using *Eucalyptus* spp. (also known as Blue gum tree) leaves is reported to be among the most commonly exploited home remedies (Paudyal *et al.*, 2022). Besides, evidence shows that

multi-vitamins, herbs and nutritional supplements present in most Complementary and Alternative Medicines (CAM), either help in boosting the immune system or offer protection from infection (Charan *et al.*, 2021). Research has also shown that natural products with proven therapeutic or antiviral activity have been a cheap and safe means to treat disease (Al-Naggar *et al.*, 2021).

In the first quarter of 2020, at the onset of the COVID-19 pandemic, there was a sudden increase of 14.4% in ginger exports globally attributed to its use in herbal remedies (Ayipey, 2020). Studies suggest steam inhalation as one of the means to mitigate COVID-19 symptoms with particular inhibitory efficacy against the virus (Gowrishankar *et al.*, 2021) via other demonstrated curing herbal effects. Eucalyptus essential oils vapor inhalation has also been suggested as a concomitantly safe and hygienic procedure with the benefits of reducing muscle pain, cough and dyspnea; common symptoms of COVID-19 (Abbass, 2020). Additionally, in Morocco, the most frequently used medicinal plant part was the leaves at 28.43% and the *Eucalyptus* spp. was ranked first at Manufacturing Unit Value (MUV) = 0.967 (Chaachouay *et al.*, 2021).

Fundamentally, Traditional Medicine Practitioners (TMP) have made use of *Eucalyptus* spp. leaves in trying to treat respiratory ailments (Tabuti *et al.*, 2010). At the onset of the COVID-19 pandemic, use of herbal remedies like the *Eucalyptus* spp. was heavily studied. Areas that utilized such remedies appeared to have lower mortality rates (Ali Al-Garawyi *et al.*, 2020a).

2.4.3 Steam inhalation

It has been observed that temperatures of steam could denature the capsid of the SARS-CoV-2 envelope (Sarker, 2021). Studies have demonstrated that through heat inactivation, detection rates of SARS-CoV-2 are reduced to a large extent (Zou *et al.*, 2020). Stability/decay of the SARS-CoV-2 on nonporous surfaces under a range of temperatures has also been reported (Biryukov *et al.*, 2020).

Steam inhalation has been reported as a potential treatment for COVID-19 (la Marca *et al.*, 2021). However, only limited details have been availed on the thermodynamic inactivation of the SARS-CoV-2 virus under steam inhalation. More so, there has been inadequate

information regarding the significance of the use of herbal remedies during this practice.

Studies prospect that steam inhalation could be an important home remedy, especially in clinical stages 1 and 2, relieving patients of congestion and loosening thick mucus (Sarker, 2021). With heat being its quintessential facet, steam inhalation could have a significant impact on the virulence of SARS-CoV-2. This could bear an intuitive outlook on viral incapacitation in the preliminary stages of infection. However, practice-based evidence of the effects of steam inhalation is recommended in order to have a consolidated insight (Chowdhury et al., 2022).

Traditional medicines have become an urgently sought after solution for treatment of various respiratory diseases. For instance, *Eucalyptus* spp. was found to be among the most commonly used plant species to treat respiratory infections like Tuberculosis (TB) (Tabuti *et al.*, 2010). At the onset of the COVID-19 pandemic, Eucalyptus essential oil was among plant extracts that were studied to prevent SARS-CoV-2 multiplication via its antiviral activity (Ali Al-Garawyi *et al.*, 2020b). It is understood that the major molecular mechanisms involved are antiviral activity via direct inactivation where the monoterpenes bind free viruses on the surfaces proteins used in virus-host interactions (Mieres-Castro *et al.*, 2021). Jensenone, a cyclic ether and a monoterpenoid, is a component of Eucalyptus essential oils. Owing to its high binding affinity, Jensenone was studied to be a potential inhibitor for COVID-19 M-protein (Dev and Kaur, 2020). Some studies have shown that there was significant reduction in virus titers when *Eucalyptus globulus* essential oil was used against Herpes simplex virus type I (HSV-I). Particularly, *in vitro* experiments indicated that there was significant antiviral effect of 1,8-cineole and α-pinene against HSV-1 (Hayat *et al.*, 2015). However, *Eucalyptus sideroxylon* leaves potency was greatest against HSV-II when they were exploited the leaf extracts on Vero cells (Okba *et al.*, 2017).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study design

This study was experimental (Creswell and Creswell, 2018) involving non-dichotomous data.

3.2 Study site and study sample size

The sample size for steam inhalation temperature determination was calculated using the formula for continuous variables.

$$N = \frac{2(Z_{(1 - \alpha/2)} + Z_{\beta})^2 \times \sigma^2}{\Delta^2}$$

Where;

- N = is the sample size for a continuous variable/data
- α = Significance level at 0.05
- β = Power, probability of detecting a significant result at 80%
- σ = Standard deviation of the data at 0.25
- Δ = Minimal effect of interest (at 0.25); and
- Z = Refers to points on a normal distribution to the required power (β) and significance level (α) given above.

$$N = \frac{2}{0.25^2}$$

$$N = \frac{2}{0.0625}$$

Sample Size (N) = 16

However, Slovin's formula (Stephanie, 2020) was used to give an accurate approximation with regards to the limited data concerning temperature profiles attained during steam

inhalation in home remedy practices. Therefore, adjusting to the final minimal sample size where;

N - Total population = 16

e - Margin of error = 0.25

$$n = \frac{N}{1 + N\mathrm{e}^2}$$

$$n = \frac{16}{1 + (16 \times 0.25^2)}$$

Sample size n = 8 participants were used in the study.

The participants were recruited from Lusaka city but within a radius of 2.5 Km around the University of Zambia (UNZA) main campus. Random individuals were approached and asked to participate in the study by undergoing a single 20-minute steam inhalation session on voluntary basis.

3.3 Eligibility criteria for participants

3.3.1 Inclusion criteria

Both male and female volunteers were recruited into the study provided they were at least 18 years of age; tested negative for COVID-19 Rapid Diagnostic Test (RDT); were not hypertensive, diabetic or asthmatic; did not have any respiratory disease and; did not have a fever/cough.

3.3.2 Exclusion criteria

Potential volunteers for the study could not be recruited into the study if they were below the age of 18 years; tested positive for COVID-19 RDT; were hypertensive; were diabetic; had any respiratory disease and; anyone who was undergoing drug therapy, especially for COVID-19.

3.4 Experimental approach

3.4.1 Participant screening

Before any volunteer could participate in the study, they were required to read and

understand the participant information sheet (PIS) (Appendix B) and the informed consent form (Appendix C) before signing it. For volunteers with difficulties understanding the PIS and the consent form, the principal investigator (PI) endeavored to explain it to them before they could be allowed to participate in the study.

Each volunteer was swabbed for one nasopharyngeal sample from UNZA clinic by the medical staff and a COVID-19 RDT was done (Sure Status®, Premier Medical Corporation). The COVID-19 test was free of charge on the part of the potential participant but provided by the UNZA clinic. Potential participants who returned a negative COVID-19 test were further screened for other illnesses using the health checklist and selection criteria (Appendix D) and as deemed appropriate by the Medical Officer at the UNZA clinic. Potential participants meeting these criteria were finally recruited to participate in the study. The steam inhalation session per participant was done within 48 hours of returning a negative COVID-19 test. The participant was allowed to choose the time of undergoing steam inhalation between 09.00 hours and 16.00 hours in line with the opening hours of the UNZA clinic.

3.4.2 Determination of temperatures attained during steam inhalation

The steam inhalation procedure commonly called *Ukufutikila* in Bemba was set up to mimic the practice in home remedies through the creation of a microenvironment. This was made possible by placing a blanket over a participant using a fabricated steel frame (Figure 3.1). However, steam inhalation sessions were conducted both with and without participants and the temperatures attained in both were conclusively compared. All the further experiments in this study used the temperature profiles obtained from steam inhalation sessions with participants.

The steel frame ensured adherence to the safe distance between the participant and the hot water. It also allowed strategic placement of the thermometer for temperature monitoring with reduced risk of breaking the microenvironment. Breaking the microenvironment would compromise the temperatures established within. Participants were not in direct contact with the boiled water. They sat 5 cm away from the hot water and bent forward at about 30 cm from the surface of the boiled water while sitting on a 40 cm-high stool depicted in Figure 3.1. The steam in the microenvironment was generated using 13 *l* of

water boiled for approximately 50 minutes to an average of 94.7˚C before the participant was allowed into the microenvironment. The temperature of steam was allowed to drop through heat exchange with the participants. There was no attempt to maintain the temperature at specific values. The drop in temperature of the steam in the microenvironment was measured using a conventional 100˚C thermometer. The thermometer, suspended on a clamp, was placed at a height not exceeding 8 cm from the surface of the boiled water.

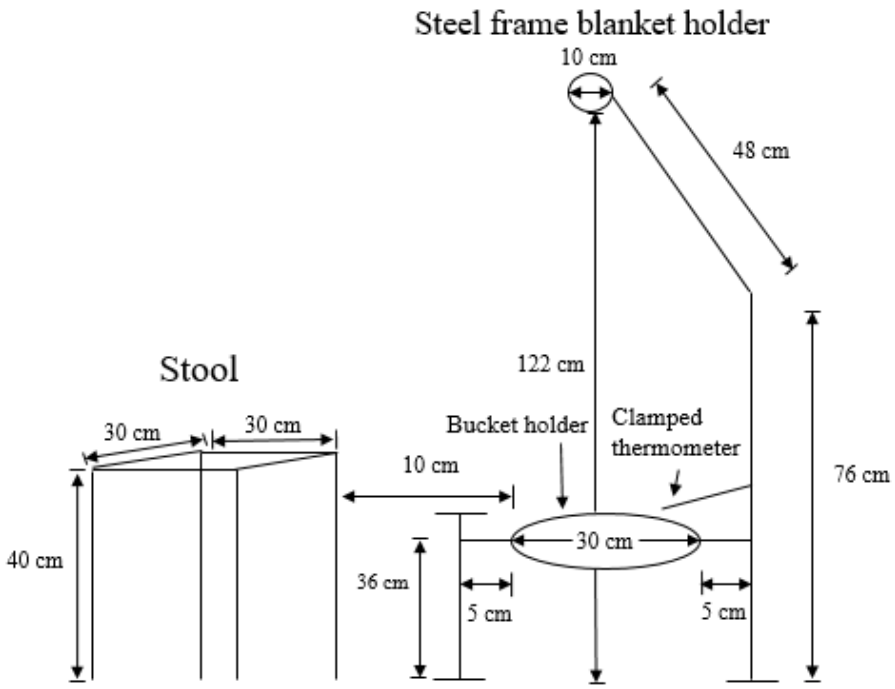

Figure 3.1. Fabricated steel blanket holder and stool.

Temperature drops in the microenvironment were monitored for 20 minutes in line with other studies (la Marca *et al.*, 2021) at two-minute intervals. Steam inhalation sessions were conducted under the close supervision of medical staff from the UNZA clinic and the PI. The temperatures obtained in this experiment were used in the subsequent experiments.

3.4.3 Effect of temperatures attained during steam inhalation on glycoprotein activity

To obtain baseline data on temperatures for use in heat inactivation of SARS-CoV-2 in the *in vitro* infection of Vero T2 cells, the established temperatures in section 3.4.2 were used

to heat treat ALP before conducting the enzymatic assays. The activity of the enzyme at the established temperatures was used to estimate whether these temperatures could inhibit ALP activity reversibly or irreversibly. This could suggest whether the heat denaturation of the glycoprotein was permanent or not.

To achieve this, a commercial Alkaline Phosphatase, Di-ethanolamine Detection Kit – AP0100 (Sigma-Aldrich) was used. Lyophilized ALP enzyme was reconstituted in 1000 µl of cold reaction buffer (1.0 M Diethanolamine, 0.50 mM and Magnesium Chloride at pH 9.8) and 30 µl of ALP was diluted to 200 µl with the reaction buffer to a final concentration of 0.15 units/ml. ALP substrate, *p*-nitrophenyl phosphate (pNPP), was prepared fresh every time an assay was carried out by dissolving 247 mg of pNPP in 1000 µl ultrapure water to give a final concentration of 0.67 M.

Enzyme assays were carried out by initially setting up the controls. The negative control was set up by mixing 980 µl reaction buffer and 20 µl of 0.15 unit/ml ALP solution, both of which were equilibrated at 37°C for 5 minutes before mixing. The positive control was set up first by equilibrating at 37°C for 5 minutes, 20 µl ALP, 960 µl reaction buffer and 20 µl pNPP before mixing the three to a final volume of 1 ml. All solutions were equilibrated in 1 ml Eppendorf tubes before mixing the contents as per assay into pre-warmed cuvettes at 37°C for 5 minutes.

Initially specific temperature means determined at time zero, 10 minutes and 20 minutes were tested for specific temperature effects. Three 20 µl of 0.15 unit/ml ALP, were thus heat treated at 56.4°C, 67.8°C for 10 minutes and at 94.7°C for 2 minutes on a heating block (Major Science, MC-203) using 1 ml Eppendorf tubes. Twenty microlitres of heat-treated ALP, 960 µl reaction buffer and 20 µl pNPP solution were then equilibrated for 5 minutes before mixing in pre-warmed cuvettes at 37°C for 5 minutes.

To replicate the temperature profiles as determined during steam inhalation and establish the effect of these temperature profiles on ALP activity, 20 µl of 0.15 unit/ml ALP was exposed to the temperatures determined during steam inhalation (Table 3.1) at time intervals of 2-minutes for a period of 20 minutes using a thermocycler (LifeECO, TC-96/G/H(b)C). The enzymes so treated were then equilibrated at 37°C for 5 minutes in 100

µl PCR tubes. The equilibrated enzyme was then mixed with 960 µl reaction buffer and 20 µl pNPP also equilibrated at 37°C for 5 minutes in separate 1 ml Eppendorf tubes before mixing in pre-warmed, 37°C for 5 minutes, 1 ml cuvettes.

The enzyme activity was followed spectrophotometrically by measuring the absorbance of UV at 405 nm by the para-nitrophenol resulting from the dephosphorylation of pNPP by the enzyme. The absorbance values were recorded over a period of five minutes immediately after enzyme inactivation, after 24 hours, 48 hours and after 72 hours of enzyme inactivation using a spectrophotometer (Optima®, SP-300). However, aliquots of the enzyme thermally exposed to 56.4°C were run immediately after incubation, after 1 hour and after 2 hours and compared to the positive control. All the enzyme activity assays were done in three replicates.

Table 3.1. Shows ALP enzyme inactivation using a thermocycler following mean temperature profile attained in the steam inhalation session with participants.

Step	**Minutes from start of steam inhalation**	**Temperature (°C)**	**Time interval (minutes)**
1	0	94.7	2
2	2	62.6	2
3	4	61.6	2
4	6	60.4	2
5	8	59.6	2
6	10	58.8	2
7	12	58	2
8	14	56.9	2
9	16	55.5	2
10	18	54.9	2
11	20	54.4	2

Enzyme activity was calculated using the following formula; -

$$Enzyme\ activity = \frac{(\Delta A_{405nm}/\text{min Test} - \Delta A_{405nm}/\text{min Blank})\ (df)\ (V_F)}{(18.5)\ (V_E)}$$

Where;

- df = Dilution factor
- V_F = Volume (in mL) of assay
- 18.5 = Millimolar extinction coefficient of pNPP at 405 nm
- V_E = Volume (mL) of sample solution used

3.4.4 *E. saligna* fresh leaves hydrodistillation

The extraction of the crude extract was done using the *Eucalyptus saligna* Sm. (accession no. 22478 – UZL) tree leaves obtained from the University of Zambia Goma Lakes plantations 15°23'22.5"S 28°19'48.8"E, Lusaka. Eucalyptus leaf extraction was done by hydrodistillation (Immaroh *et al.*, 2021; Maghsoodlou *et al.*, 2015) using a Clevenger apparatus. Fifteen grams of chopped fresh leaves and 300 ml water were placed in a round-bottomed flask and boiled. After heating to boiling (attaining a 105°C temperature), the volatile constituents were collected over a period of 20 minutes. The condensed liquid was collected in a round-bottom flask and stored in 1 ml Eppendorf tubes at – 20°C before being used for further experiments.

3.4.5 Cultivation of Vero T2 cells

The Vero T2 cells were provided by the International Institute for Zoonosis Control, Hokkaido University and the Hokudai Centre for Zoonosis Control in Zambia, University of Zambia. Initially, Vero T2 cells were first activated by mixing 1 ml of the thawed solution of banked cells into 5 ml Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 5 ml L-glucose, 6 ml D-glucose and 5 ml Pen-Strep. Media containing Vero T2 cells was then centrifuged at 810 x g (at 4°C) for 3 minutes and the supernatant discarded. The pellet was re-suspended in fresh media and transferred to new 15 ml culture flasks (Falcon®, T75). The Vero T2 cells were then placed in the incubator (Heracell™, 150i CO_2 incubator) at 37°C in 5% CO_2 and allowed to attain a growth confluency of 90%. The cells were passaged 3 times to establish a homogeneous population of cells. For the purpose of cell culture propagation, cell passage was performed three (3) times.

The passaging of cells was done using sterile culture flasks and media. Firstly, the

confluence and health of cells was checked in the original culture. Thereafter, old culture medium was aspirated and the adherent cells were washed using 5 ml Phosphate-buffered saline (PBS). In order to detach cells, a detachment solution, trypsin (0.5 ml) was used. After the cells dissociated following a 10-minutes incubation at 37°C in 5% CO_2, fresh DMEM was used to neutralize the detachment solution. The cell suspension was collected and transferred to a sterile 15 ml falcon tube. The suspended Vero T2 cells were then centrifuged then re-suspended in fresh DMEM before being transferred to a new sterile culture flask (15 ml) and incubated at 37°C in 5% CO_2.

3.4.6 *E. saligna* fresh leaves crude extract cytotoxicity tests

To determine the cytotoxicity of the *E. saligna* fresh leaves crude extract, the Vero T2 cells were exposed to the extract. A two-fold serial dilution was used in 3 wells of the 6-well plate with the highest point of dilution being 30% in culture media in line with other studies (Vimalanathan and Hudson, 2014). The cytotoxic concentration (CC_{50}) was defined by the concentration of the extract that reduced the number of viable cells by 50%. The non-cytotoxic concentration was also obtained using the same method (Schnitzler, 2019). Only the non-cytotoxic concentration of the *E. saligna* fresh leaves crude extract was used for further experiments involving the crude extract. Cell viability was assessed by scoring cytopathic effect (CPE) in each well observed under a microscope in comparison with the negative control. CPE was observed at a magnification of 100x. The CPE comprised of cell death observed from floating cell debris and reduction in cell confluency. In addition, CPE was observed from cell morphological changes like rounding of cells and elongation of cells.

3.4.7 Propagation of SARS-CoV-2 and viral load estimation

After the cytotoxicity tests (CTT), the Vero T2 cells that were not exposed to the crude extract were passaged to a new culture flask in fresh DMEM and allowed to grow. The adherent cells were grown to 80% confluency before being inoculated with the SARS-CoV-2 (αvariant) for virus propagation. The α-variant of SARS-CoV-2 was used as it was the circulating variant and latest to be isolated at the time of the study. To prepare virus stocks, archived positive SARS-CoV-2 clinical sample in virus transport media (VTM) was first centrifuged. The supernatant was applied to 10 wells containing adherent Vero T2 cells at

80% growth confluency and incubated at 37°C within 5% CO_2 for 72 hours. Upon observation of CPE, 1 ml of the culture supernatant was harvested from each well and pooled into one tube. The viral load was estimated by real-time Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR) after RNA extraction compliant with prior research (Peacock *et al.*, 2021) as described below.

Firstly, RNA extraction was done using the QIAGEN kit and according to the manufacturer's protocol. Briefly, 500 µl of the viral lysis buffer (AVL) was added to eppendorf tubes. Then 150 µl of culture supernatant was added to AVL buffer and vortexed (Vortex gene, SI-0286) for lysis. Finally, 500 µl of 95% ethanol was added to the Eppendorf tubes (for RNA precipitation) and vortexed. To filter RNA, 630 µl of the mixture was transferred to spin columns and centrifuged (TOMY, MX-207 – high speed refrigerated microcentrifuge) at 60g (at 4℃) for 1 minute. After centrifugation the supernatant was discarded and the rest of the mixture was added to the spin columns and centrifuged at 60 x g (at 4℃) for 1 minute. The resulting supernatant was discarded. Subsequently, wash buffers were used to clean the extract. Firstly, 400 µl of wash buffer 1 (AW1) was added to the spin columns. After 1 minute of centrifugation at 60 x g (at 4℃), the supernatant was discarded. Wash Buffer 2 (AW2) (400 µl) was then added to the spin columns and centrifuged at 200g (at 4℃) for 4 minutes. Thereafter, the supernatant was discarded. An elution buffer (AVE) of 60 µl to elute RNA was finally added to the spin columns and centrifuged at 60g (at 4℃) for 1 minute. The supernatant was collected in eppendorf tubes for further diagnosis using the RT-qPCR.

To run the RT-qPCR for viral load estimation, 5 µl of master mix (Macurra kit) was initially added to 20 µl PCR tubes before 2.5 µl of the purified RNA sample was added. Positive and negative controls were included. A four-step program was applied in the RT-qPCR (LightCycler® 96, 15444) for the detection of SARS-CoV-2: incubation step at 55℃ for 15 minutes, 1 cycle; activation step at 95℃ for 15 minutes, 1 cycle; Two – step amplification (40 cycles) consisting of denaturation on 94℃ 5 seconds, annealing on 60℃ for 15 seconds, elongation on 72℃ for 15 seconds; cooling step at 55℃ for 15 minutes, 1 cycle. The FAM channel was used to detect the open reading frames (ORFs) gene while the Cy5 channel was used to detect the nucleocapsid (N) gene. The VIC channel was used as the internal

control whereas the Texas Red channel was used to detect the envelope (E) gene. The results were read using LightCycler® 96 software. A Cycle threshold (Ct) value less than or equal to 37 had a positive call and inversely. The lower the Ct-values, the higher the viral load in each sample. To ascertain viral growth and replication, RNA isolation and quantification by RT-qPCR was performed before inoculation, followed by subsequent assessments at 24, 48 and 72 hours post inoculation.

To see the effects of heat and *E. saligna* fresh leaves crude extract on SARS-CoV-2 *in vitro* infectivity, the Vero T2 cells were inoculated in three ways. Firstly, the Vero T2 cells were inoculated with SARS-CoV-2 material in the presence of the crude extract. Secondly, with heat inactivated SARS-CoV-2 material only. Finally, the Vero T2 cells were inoculated with heat inactivated SARS-CoV-2 material in the presence the crude extract. The positive controls used in the subsequent sections were Vero T2 cells exposed to SARS-CoV-2 material. While, the negative controls were Vero T2 cells not exposed to neither the SARS-CoV-2 material or crude extract.

3.4.8 SARS-CoV-2 infectivity of Vero T2 cells in the presence of *E. saligna* fresh leaves crude extract

To determine SARS-CoV-2 infectivity of the Vero T2 cells in the presence of *E. saligna* fresh leaves crude extract, 0.5 ml of infectious material was added to 80% confluent Vero cells. Successively, the crude extract was added to the culture at a non-cytotoxic concentration of 13% in culture. The Vero T2 cells were then placed in a 5% CO_2 incubator at 37°C for 72 hours in the BSL-3 laboratory. To monitor virus growth, 200 µl of the culture supernatant was harvested at 24, 48 and 72 hours post inoculation (Sasaki *et al.*, 2021) for diagnosis to obtain Ct-values using RT-qPCR (section 3.4.7). Finally, the CPE of the SARS-CoV-2 on the Vero T2 cells was observed 72 hours post inoculation and compared to the controls. The CPE was observed under the microscope as described in section 3.4.6.

3.4.9 Heat inactivated SARS-CoV-2 infectivity of Vero T2 cells

The SARS-CoV-2 infectious material was thermally treated according the established temperature profile. Pre-thermal treatment of SARS-CoV-2 infectious material before infecting Vero T2 cells, was done to establish whether steam inhalation can be applied as a prevent measure, especially to those who could have been exposed to the virus for one

reason or another. This was to determine the effect of the temperature profile attained during steam inhalation as guided by ALP activity on SARS-CoV-2 *in vitro* infectivity. Precisely 5 ml of the SARS-CoV-2 infectious material from the pooled sample was exposed to the established temperatures (Table 3.1) on the heating block (Scinics, ALB-301). Thereafter, 0.5 ml of heat inactivated SARS-CoV-2 material was inoculated to 80% confluent Vero T2 cells. The culture supernatant (200 µl) was harvested at 24, 48 and 72 hours post inoculation to obtain Ct-values for virus growth estimation. The CPE was observed at 72 hours as in section 3.4.6.

3.4.10 Combined effect of heat inactivation and *E. saligna* fresh leaves crude extract on SARS-CoV-2 infectivity of Vero T2 cells

Furthermore, the effect of temperature on SARS-CoV-2 *in vitro* infectivity in the presence of *E. saligna* fresh leaves crude extract was determined. Initially, *E. saligna* fresh leaves crude extract at a concentration of 13% in culture, was added. Thereafter, 0.5 ml of heat inactivated SARS-CoV-2 material was inoculated to 80% confluent Vero T2 cells. Culture supernatant (200 µl) was harvested at 24, 48 and 72 hours post inoculation to obtain Ct-values for virus growth estimation. As in the other cultures discussed above, the CPE was observed at 72 hours (section 3.4.8).

3.5 Data analysis

Descriptive and inferential statistics were used to analyze data in this study. Data collected were entered into Microsoft Excel and analyzed using GraphPad Prism (version 9.0). All of the graphs were generated using GraphPad Prism (version 9.0) unless otherwise stated. Steam inhalation temperature values of the same time points were described by mean and standard error of mean (mean ± SEM). This data was then subjected to a Kolmogorov-Smirnov test to test for normality. The steam inhalation temperatures were compared using Analysis of Variance (ANOVA) and the T-test. Furthermore, ANOVA was used to compare ALP enzyme activity. The CTT of *E. saligna* fresh leaves crude extract was analyzed using non-linear regression. Finally, the viral loads of SARS-CoV-2 infectious material were estimated using mean ± SEM of the Ct-values and further compared using the T-test and ANOVA. All statistics were considered significant at $p \leq 0.05$.

3.6 Ethical clearance

The study was carried out with ethical approval obtained from the Excellence in Research Ethics and Science (ERES) Converge Institutional Review Board (IRB) committee in Lusaka under reference 2022-Mar-019 (Appendix A) and approved by the National Health Research Authority (NHRA) under registration number NHRAR-R-1390/17/11/2022 (Appendix F). Additionally, the issues requiring ethical clearance have been clearly stated in Appendix B. Finally, permission was obtained from the UNZA clinic to conduct the steam inhalation experiments at the premises with monitoring of the sessions and participant screening by the Clinic's Medical Officer (Appendix E).

CHAPTER FOUR

4.0 RESULTS

4.1 Demographic characteristics of participants

The eight participants recruited to the study were aged between 18 and 36 years with 1 female and 7 males. The participants' body weights ranged from 55 Kg to 93 Kg (Table 4.1).

Table 4.1. Demographic characteristics of the eight volunteer participants.

Participant No.	**Sex**	**Age (years)**	**Weight (Kg)**
1	Male	28	56
2	Male	34	87
3	Male	31	65
4	Male	32	93
5	Male	36	70
6	Male	25	63
7	Female	28	55
8	Male	18	61

4.2 Determining temperature profiles in the participant-free microenvironment.

Eleven mean temperature values were obtained over a period of 20 minutes (Figure 4.1). This was done every two minutes from minute zero to minute 20 in the participant-free microenvironment. The highest temperature value (95.2°C) was obtained at minute zero which quickly dropped to 65°C within 2 minutes. Temperatures progressively reduced until reaching an average temperature value of 56.7°C at the 20^{th} minute. There was a significant difference (ANOVA: $p < 0.001$) in mean temperature values obtained.

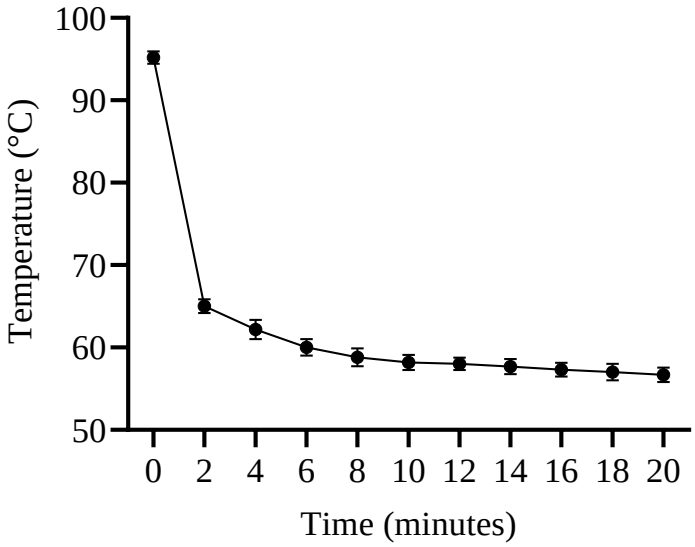

Figure 4.1. Shows a progressive reduction of determined temperature profile tenable in steam inhalation without participants. The values shown are mean ± SEM of triplicate steam temperature drops monitored over 20 minutes.

4.3 Temperature profiles tenable in steam inhalation

Eight participants each underwent a single 20-minute steam inhalation procedure (Figure 4.2). The mean temperatures were calculated for the 8 temperature values at each time point obtained from each participant's steam inhalation session. The data was normally distributed as the test statistic (0.1541) obtained was less than the critical value (0.40925) at $\alpha = 0.05$. Furthermore, there was a significant difference in the means obtained (ANOVA: $p < 0.001$).

The highest mean temperature value was determined at minute zero to be 94.7°C. The lowest mean temperature value at the 20^{th} minute was determined to be 54.4°C. The means of the temperature profiles attained with participants were compared to the range of temperatures obtained in the participant-free microenvironment (Figure 4.1). There was no significant difference (T-test: $p = 0.8706$) between the two sets of temperature values.

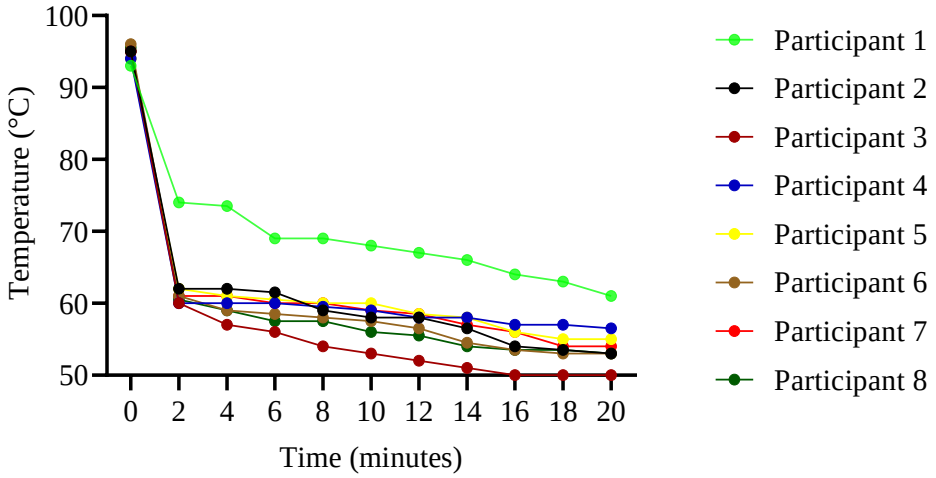

Figure 4.2. Determined temperature profiles tenable in steam inhalation with participants. The values shown are temperature values at each time point obtained from each participant's steam inhalation session.

4.4 Thermal inactivation of ALP at 56.4°C

To determine effects of specific temperatures attained during steam inhalation on ALP activity, specific temperatures were obtained at three time points. The lowest average temperature was attained in the last 10 minutes of the steam inhalation sessions to be 56.4°C (Figure 4.3). There was no difference in the enzyme activity between the positive control (7.207 ± 0 µmol/min) and that of the ALP enzyme (7.207 ± 0 µmol/min) thermally treated at 56.4°C. This clearly indicated that ALP was not affected by exposure to 56.4°C heat for 10 minutes.

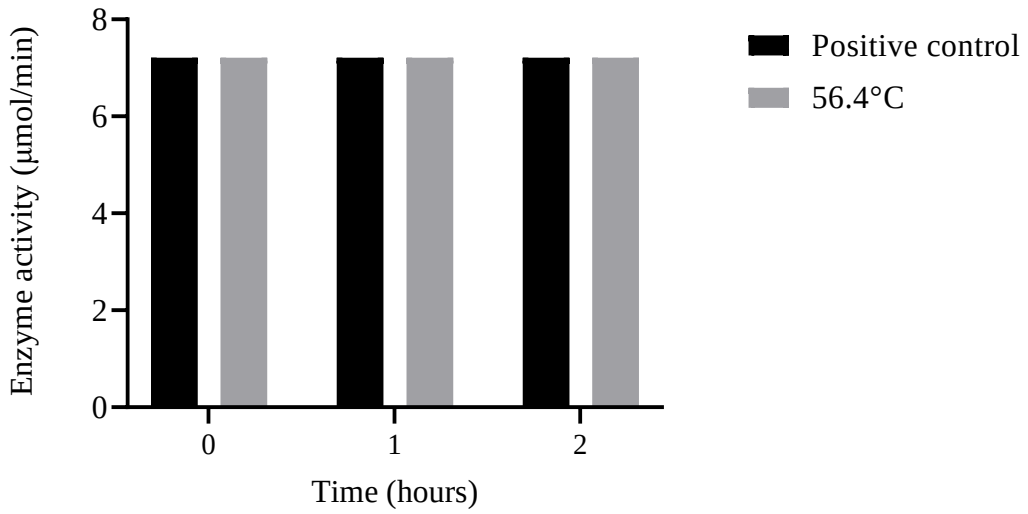

Figure 4.3. Shows the enzymatic activity of ALP enzyme aliquots exposed to 56.4°C and positive controls. The values shown are means of triplicate samples.

4.5 Thermal inactivation of ALP at 67.8°C

An average temperature of 67.8°C was obtained in the first 10 minutes of the steam inhalation sessions. The ALP enzyme assays carried out after 24 hours, after 48 hours and after 72 hours of incubation enzyme after inactivation at 67.8°C showed reduced enzyme activity (0.054 ± 0.010 µmol/min, 0.332 ± 0.012 µmol/min and 0.187 ± 0.022 µmol/min) from the enzyme assay carried out immediately after heat exposure (0.706 ± 0.073 µmol/min) (Figure 4.4). However, the enzyme activities at 24, 48 and 72 hours after heat treatment minimally fluctuated and were significantly different (ANOVA: $p < 0.001$). The fluctuations in enzyme activity observed, indicated that ALP was able to recover from heat inactivation but with over 50% reduced activity over a period of 72 hours possibly due to reversible denaturation.

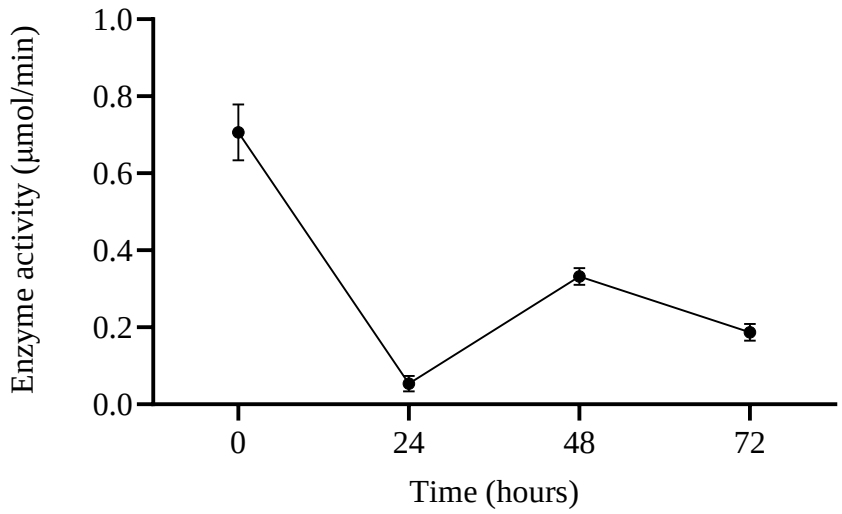

Figure 4.4. Enzyme activity of ALP enzyme exposed to 67.8°C. The values shown are mean ± SEM of triplicate samples.

4.6 Thermal inactivation of ALP at 94.7°C

The attainment of a temperature of 94.7°C, albeit for 2 minutes, piqued the interest to find out the effect of this specific temperature for the indicated duration on the activity of ALP. This would provide baseline data for use in testing its possible effect on SARS-CoV-2 *in vitro* infectivity. An aliquot of ALP enzyme was thermally exposed to 94.7°C for a period of 2 minutes which was the time it took to drop to the next temperature of the steam inhalation sessions. The enzyme activity was recorded immediately after thermal treatment, then at 24, 48 and 72 hours after thermal treatment. Enzyme activity significantly declined in the first 48 hours from 0.036 ± 0 μmol/min to 0.012 ± 0.006 μmol/min. Then notably showing increased activity at 72 hours (0.024 ± 0.006 μmol/min) (Figure 4.5). The enzyme activities were observed to be significantly different (ANOVA: $p = 0.005$).

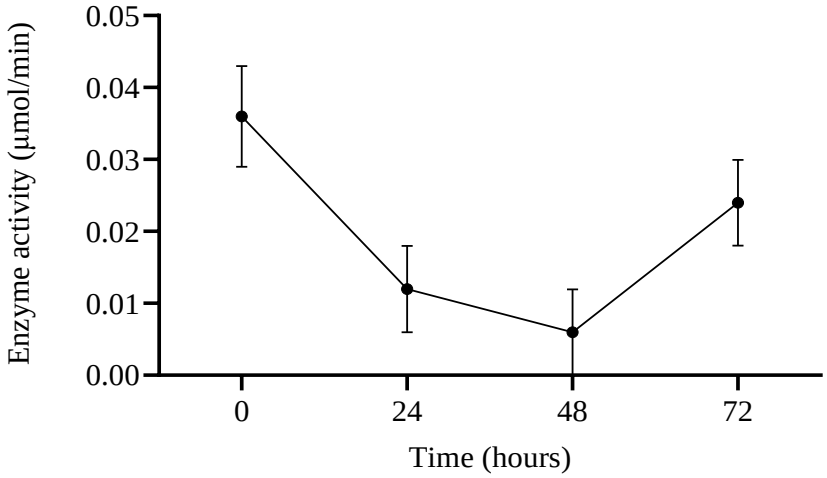

Figure 4.5. Shows ALP enzyme activity after being exposed to 94.7°C. The values shown are mean ± SEM of triplicate samples.

4.7 Thermal inactivation of ALP using the entire temperature profiles of the steam inhalation

Reversible denaturation of ALP at 67.8°C and 94.7°C following its inactivation for 10 and 2 minutes, respectively, would not be desirable if ALP were the SARS-CoV-2 glycoprotein. This would mean a possible successful interaction between the SARS-CoV-2 glycoprotein and host cells' ACE2 receptor risking an infection. However, ALP enzyme aliquots exposed to the whole temperature profile of steam inhalation for 20 minutes but once, showed no activity at 72 hours. The enzyme activity sharply reduced to 0.024 ± 0.006 µmol/min in the first 24 hours from 0.103 ± 0.013 µmol/min immediately after thermal treatment. Enzyme activity continued to reduce until there was zero enzyme activity at 72 hours (Figure 4.6). The recorded activities at the four time points were significantly different (ANOVA: $p = 0.0002$). Lack of regain of ALP enzymatic activity suggested a possible permanent denaturation of the enzyme. This set a stage for testing of this temperature profile on the actual *in vitro* infectivity of SARS-CoV-2 of Vero T2 Cells.

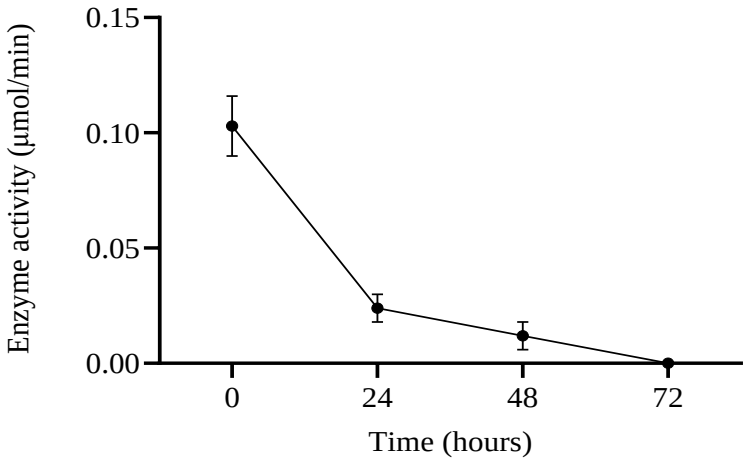

Figure 4.6. Enzyme activity of ALP enzyme aliquots exposed to 20-minute heat treatment with average temperatures obtained from an entire steam inhalation session. The values shown are mean ± SEM of triplicate samples.

4.8 Cytotoxicity test of the *E. saligna* fresh leaves crude extract on Vero T2 cells

The viability of Vero T2 cells exposed to *E. saligna* fresh leaves crude extract was observed and compared to the control. This was conducted to establish the highest concentration of *E. saligna* fresh leaves crude extract that Vero T2 cells could be exposed to without affecting their growth. The specific concentration at which dose response was provoked (CC_{50}) was found to be 16.51% of the extract in culture media. This implied that at CC_{50} = 1.2177 (16.51%), the Vero T2 cells exhibited 50% CPE (Figure 4.7). The R^2 value (0.9608) indicated a good fit of the model to the data.

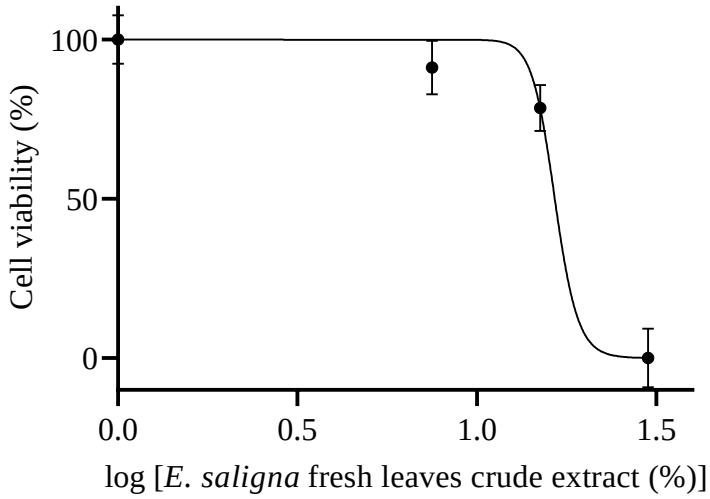

Figure 4.7. The dose response curve showing CC_{50} of the *E. saligna* fresh leaves crude extract on the Vero T2 cells at 1.2177 (16.51%) (R^2 = 0.9608). The graph was constructed using log concentrations (%) of the *E. saligna* fresh leaves crude extract and the % viability of the Vero T2 cells.

4.9 *In vitro* infectivity of heat inactivated SARS-CoV-2 in the presence and absence of *E. saligna* fresh leaves crude extract.

To see the effects of heat and *E. saligna* fresh leaves crude extract on SARS-CoV-2 *in vitro* infectivity, the Vero T2 cells were inoculated in three ways. Firstly, the Vero T2 cells were inoculated with SARS-CoV-2 material in the presence of the crude extract. Secondly, with heat inactivated SARS-CoV-2 material only. Finally, the Vero T2 cells were inoculated with heat inactivated SARS-CoV-2 material in the presence the crude extract. After 72 hours post inoculation, CPE was only observed in the positive control assays (cultures exposed to SARS-CoV-2) at 100%. However, there was no CPE observed in Vero T2 cells exposed to SARS-CoV-2 material in the presence of *E. saligna* fresh leaves crude extract, heat treated SARS-CoV-2 material and heat treated SARS-CoV-2 material in the presence of *E. saligna* fresh leaves crude extract (Figure 4.8).

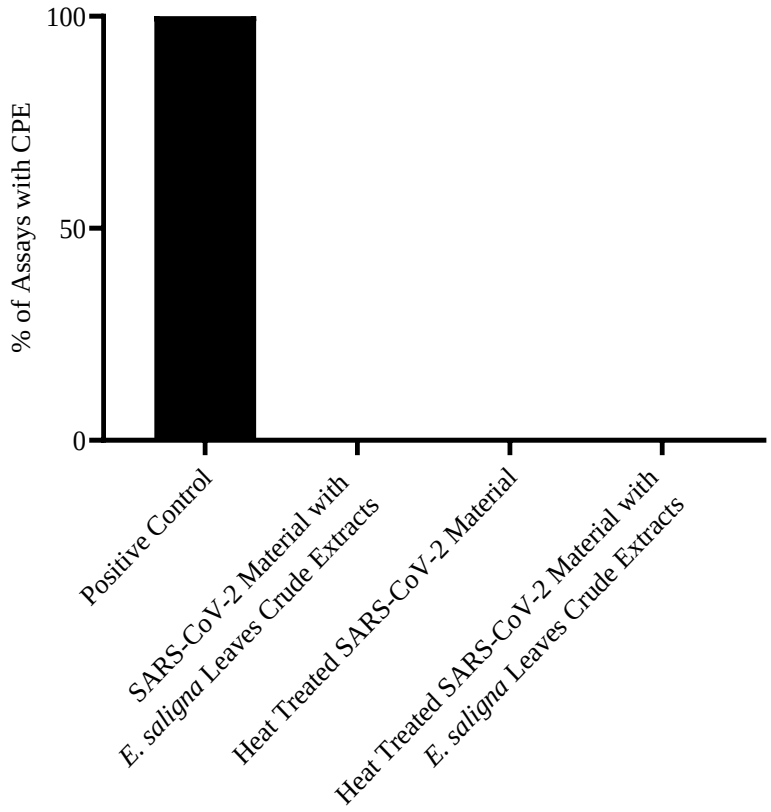

Figure 4.8. Cytopathic effects produced by SARS-CoV-2 after being exposed to *E. saligna* fresh leaves crude extract after thermal treatment of SARS-CoV-2 material and combined exposure to heat and exposure to *E. saligna* fresh leaves crude extract of SARS-CoV-2.

Furthermore, the CPE observed under the light microscope at a magnification of 100× showed large plaque formations in the positive control. Additionally, there was a visible change in cell morphology and decrease in cell confluence in the positive control (Figure 4.9) indicating virus multiplication.

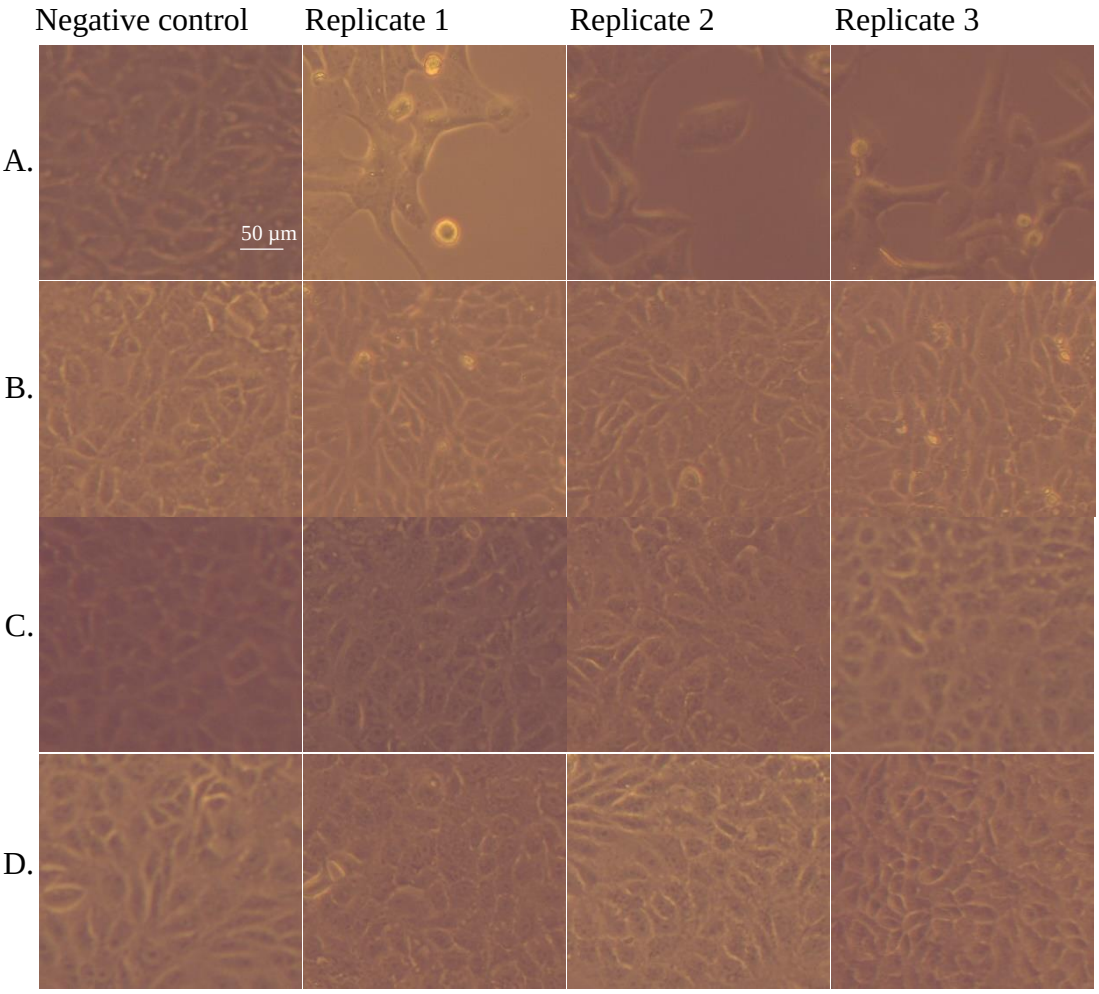

Figure 4.9. Observed CPE under the microscope (Primo Vert, T204715) after inoculation with SARS-CoV-2 material; (A) Vero T2 cells inoculated with SARS-CoV-2 material (positive control) (B) Vero T2 cells inoculated with SARS-CoV-2 material with *E. saligna* fresh leaves crude extract (C) Vero T2 inoculated with heat inactivated SARS-CoV-2 material (D) Vero T2 cells inoculated with heat inactivated SARS-CoV-2 material with *E. saligna* fresh leaves crude extract. The culture supernatant was harvested at 24, 48 and 72 hours and triplicate Ct-values calculated to estimate virus growth. The pictures were taken using a camera (Carl Zeiss Microscopy GmbH, M9K 9853 – AxioCam ERc 5s) mounted on the microscope at a magnification of 100×.

There was an increase in viral load of SARS-CoV-2 in the culture supernatant 72 hours after inoculation (C2) as compared to the viral load of the initial SARS-CoV-2 material inoculated (C1) (Figure 4.10). The viral loads were significantly different (Student T-test; $p = 0.037$). However, the mean Ct-values of the positive control and SARS-CoV-2 culture supernatant from the cultures exposed to SARS-CoV-2 material with *E. saligna* fresh leaves crude extract, heat treated SARS-CoV-2 material and heat treated SARS-CoV-2 material with *E. saligna* fresh leaves crude extract (Figure 4.10) were not significantly

different (ANOVA; $p = 0.771$) 72 hours post inoculation.

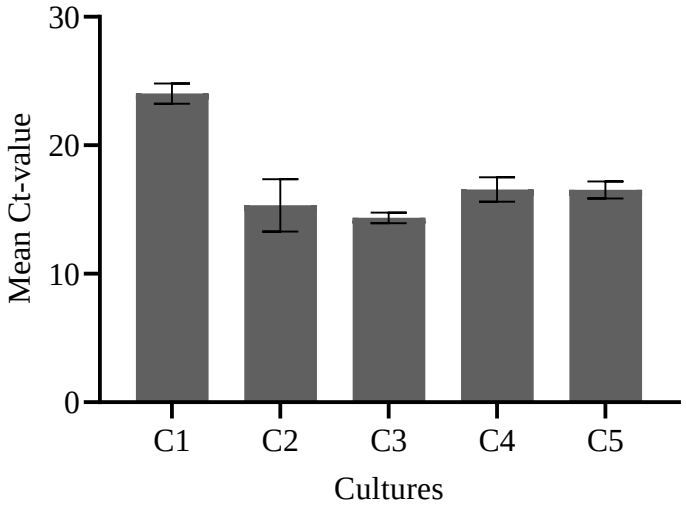

Figure 4.10. Mean Ct-values of cultures 72 hours post virus exposure (C1 - Culture 1 = SARS-CoV-2 material before inoculation; C2 - Culture 2 = Positive control (inoculated with SARS-CoV-2 material); C3 - Culture 3 = Inoculated with SARS-CoV-2 material with *E. saligna* fresh leaves crude extract; C4 - Culture 4 = Inoculated with heat treated SARS-CoV-2 material; C5 - Culture 5 = Inoculated with heat treated SARS-CoV-2 material with *E. saligna* fresh leaves crude extract

CHAPTER FIVE

5.0 DISCUSSION

5.1 Temperature profiles tenable in steam inhalation with and without participants.

Steam inhalation is among the home remedies which most communities use to treat various diseases (Anam *et al.*, 2021). At the height of the COVID-19 pandemic, the Government of Zambia devised a home based care system to manage its spread. Asymptomatic patients and those with very mild symptoms on home based care management were encouraged to use *Ukufutikila* or steam inhalation among other home remedies to relieve nasal congestion (Ministry of Health Zambia - National Public Health Institute, 2021). The benefits of steam inhalation include relief from nasal congestion, loosening the thick mucus, ease of breathing, and acceleration of virus removal. Besides, it is an inexpensive procedure (Starker *et al.*, 2021). Therefore, this study aimed at determining the temperatures attained in steam inhalation and their possible effect on the *in vitro* SARS-CoV-2 infectivity.

In this study, a range of temperatures were established in the steam inhalation setup. The trend of the temperature profiles determined entailed a progressive reduction of temperatures under the microenvironment. This trend was observed both in the participant-free setup and in that with participants. It was established in the participant-free microenvironment that a range of temperature values from an average of 95.2°C to an average of 56.7°C was attained within 20 minutes. However, in the steam inhalation setup, participants were exposed to a range of temperature values from an average of 94.7°C to an average of 54.4°C within 20 minutes. There was no significant difference (p = 0.8706) between the two ranges of temperatures established in this study from the two setups. This implied that the presence of participants did not influence the temperatures attained in the steam inhalation setup. Essentially, the maximum temperature, 94.7°C, did not exceed those that have been determined in a sauna bath (95°C) (Marvin *et al.*, 2020; Cohen, 2020). This allays fears of participants'/practitioners' safety when exposed to steam inhalation temperatures. Although the humidified air in this study was determined to have higher temperatures than other studies (Swain and Sahu, 2021; la Marca *et al.*, 2021; Chowdhury

et al., 2022), the participants did not exhibit any form of harms. The quick drop of temperature from 94.7°C to 62.6°C within 2 minutes and a further gradual decrease to 54.4°C may have guaranteed the safety of the participants. Besides, the steam inhalation session being closely supervised may have also warranted the participants' safety.

The temperatures determined in this study correspond with those (70°C – 80°C) indicated to have shown benefits against COVID-19 (Chowdhury *et al.*, 2022). Despite having distinct maximum and minimum temperatures, the temperature range determined in this study was analogous to those utilized by others (55°C – 65°C) within 20 minutes (la Marca *et al.*, 2021).

Other research, in studying the common cold, utilized the delivery of a consistent temperature (41°C) of humidified air to the upper airways of volunteers for 2 hours using devices (Hei *et al.*, 2018). This is in disparity with the current study in which participants were exposed to a range of temperatures which gradually reduced within 20 minutes. Besides, the temperatures established in this study were higher. This may have been because the humidified air in the microenvironment of the steam inhalation setup was generated differently by first heating the water to boiling. Also, this study relied on a natural drop of temperatures mimicking home setups and did not use devices to maintain a consistent temperature. This entails that steam inhalation as a home remedy does achieve adequate temperatures at almost no cost and with no need for specialized equipment. Furthermore, some studies that have conducted steam inhalation therapies have only been able to produce steam with a temperature range of 41°C – 47°C using steaming devices (Marvin *et al.*, 2020). In practice, steam inhalation periods are employed varyingly although studies have experimented with durations ranging from 5, 15, to 20 minutes (la Marca *et al.*, 2021; Mandal, 2020; Little *et al.*, 2013). Others have experimented with and confirmed that the temperature range from 42°C to 45°C drastically reduced COVID-19 symptoms (Swain and Sahu, 2021). These temperature ranges that have been reported elsewhere are indicatively lower than those established in this study (54.4°C – 94.7°C) as is in steam inhalation performed as a home remedy. Which would imply that the temperatures determined in this study may have better effects on the COVID-19 symptoms.

5.2 Effect of temperatures determined during steam inhalation on ALP *in vitro* activity

The steam inhalation temperatures determined (section 5.1) were used to approximate their effect on ALP activity, a surrogate of SARS-CoV-2 membrane spike protein. As such, ALP was first chosen for this purpose because it is a glycoprotein like SARS-CoV-2 membrane spike protein that determines a successful infection of host cells. Secondly, its thermal stability (Sharma *et al.,* 2014) lies within the temperatures indicated to be beneficial in inhibiting SARS-CoV-2 infection (la Marca *et al.*, 2021). Thirdly, ALP assays enabled direct observation of whether the temperatures attained in steam inhalation would indirectly lead to the reversible or irreversible heat inactivation of SARS-CoV-2.

This study observed fluctuations in enzyme activity in the ALP assays exposed to 67.8°C for 10 minutes and 94.7°C for 2 minutes. Specifically, at these temperatures, reduced enzyme activity was followed by resumption of enzyme activity from the signs of recovery observed. This resumption in enzyme activity was observed after subjecting the ALP aliquots to 67.8°C and 94.7°C although at different lengths. These fluctuations observed both indicate reversible denaturation that the ALP enzyme may have been subjected to after heat treatment at these specific temperatures. The effect of inactivation of ALP at 94.7°C for 2 min was not immediately apparent. However, it was observed at 24 hours and 48 hours with recovery of more than 50% activity at 72 hours suggesting a possible reversible denaturation. At a high temperature of 94.7°C, the resumption of activity may be attributed to the quick drop in temperatures within a short period. This may also be attributed to the heat treatment at 67.8°C as other studies have exposed the glycoprotein specific temperatures at longer periods as earlier mentioned (Hei *et al.,* 2018).

The ALP enzyme activity after heat treatment at 56.4°C did not differ from the positive control. The baseline data in this study revealed that the ALP glycoprotein did not lose its activity after thermal treatment at 56.4°C for 10 minutes. This entailed that there was no denaturation of the glycoprotein at 56.4°C. This could have been as a result of the time-temperature aspect. Moreover, it has been justified that there could be a crucial relationship between time and temperature in ALP thermal treatments (Holm *et al.*, 2022). Other studies (Rankin *et al.,* 2010) have shown that ALP denaturation could occur between 50 to 60°C for 60 minutes. The length (10 minutes) of the 56.4°C thermal treatment of the present

study contrasted with the assays discussed above which have had longer periods of heat treatment within the same temperature range. Therefore, the difference seen in this study can be ascribed to the period of heat treatment.

Taken together, the results about the specific temperature exposures for a short time of less than or equal to 10 minutes of ALP, suggested reversible denaturation of ALP and subsequent regaining of enzyme activity. These results are consistent with the response of ALP'S thermal stability which are dependent on time of thermal exposure (Sharma *et al.*, 2014). In terms of SARS-CoV-2 membrane spike protein, this would suggest a latent period for the virus that would wake to achieve a possible entry or infection of a host cell. It has been reported that SARS-CoV-2 can thermally be inactivated at single temperatures for different time durations that were not consistent with the time durations of the measured temperature profiles of steam inhalation (*Ukufutikila*) (Barrow *et al.*, 2021; Batéjat *et al.*, 2021; Patterson *et al.*, 2020). However, steam inhalation as a home remedy (as is in *Ukufutikila*) does not use single temperatures. It is for this reason that these temperatures of inactivation for shorter periods of less than or equal to 10 minutes failed to provide baseline data to further pursue their possible effect on SARS-CoV-2 infectivity of Vero T2.

In order to mimic steam inhalation, determination of the effects of temperature on ALP was carried out using the whole temperature profile from the steam inhalation. Therefore, the ALP heat treatment followed the temperature profile obtained in the steam inhalation (section 4.3). The thermal inactivation using the entire temperature profile of the steam inhalation showed no resumption of enzyme activity after 72 hours of incubation. This permanent inactivation may be attributed to the longer duration (20 minutes) of thermal treatment as compared to specific thermal treatments which were shorter as stated earlier. Interestingly, the 3 days it took to completely lose activity is less than the average incubation period of 4.96 days for SARS-CoV-2 variants (Delta and Omicron) which were more infective and highly transmissible (Galmiche *et al.*, 2023). Moreover, it is a crucial period when an estimated quarter of infected individuals would shed the infectious virus before the onset of symptoms (Hakki *et al.*, 2022). The results clearly showed complete loss of activity possibly due to irreversible denaturation of ALP. It is for this reason that this result provided the baseline data which was used to study *in vitro* SARS-CoV-2 infectivity of Vero T2 cells.

5.3 Effect of heat inactivation on *in vitro* SARS-CoV-2 infectivity of Vero T2 cells

Guided by the baseline data (section 5.2), the effect of temperature profile determined in the steam inhalation setup on SARS-CoV-2 infectivity of Vero T2 cells was determined. The heat treatment was done according to the temperature profile which irreversibly denatured the glycoprotein (ALP). This would help in approximating whether the spike glycoprotein on the SARS-CoV-2 virus would be irreversibly denatured by the temperature profile used and thus render it ineffective to infect cells.

Overall, the temperatures determined in this study lie within those that have been reported to inactivate the SARS-CoV-2 virus (Leta *et al.*, 2022). Studies have suggested that shortly after contamination, steam inhalation therapy at 55 – 65°C could minimize severe infection development (Chowdhury *et al.,* 2022). Research has established that the NTD is destabilized at 85°C (Martí *et al.*, 2021) and that steam inhalation can also denature the capsid protein of the SARS-CoV-2 envelope (Starker *et al.,* 2021). Other studies have found that at 74.8°C, the SARS CoV-2 main protease underwent thermal fluctuations which led to α-helix and β-sheet degradation at domain I and domain II, respectively (Venugopal *et al.,* 2022).

Temperature has been studied to be an important factor influencing virus infection in respiratory infections because it creates a hostile environment that compromises virus stability (Cicconetti *et al.*, 2021). SARS-CoV-2 glycoprotein denaturation is dependent on the environment that it resides in. Substantially, it has been studied to be denatured at lower temperatures (70°C) in wet conditions as compared to dry conditions (140°C) (Hernández-Arteaga *et al.*, 2022). The SARS-CoV-2 used in this study was borne in supernatant (wet environment) and overall temperature achieved was just less than 70°C. However, the steam inhalation (*Ukufutikila*) temperature profile determined in this study was able to inhibit SARS-CoV-2 infecting Vero T2 cells *in vitro*. This is due to supposedly irreversible denaturation of SARS-CoV-2 spike which is crucial to the virus entry into mammalian cells. Other studies observed complete inactivation of the virus at 75°C as compared to low infectivity at 56°C and 60°C (Scheller *et al.,* 2020). The findings of this study are therefore in line with other studies that have shown thermal inactivation of the glycoprotein. According to studies, temperature together with pH are innate physiological barriers of

infections (Cicconetti *et al.*, 2021). Notwithstanding, this study did not delve into determining the interactions of temperature with other factors.

Indeed, a lot of COVID-19 patients, arguably those exhibiting mild symptoms, have expressed a firm conviction that steam inhalation therapy helped with their recovery (Ahmed *et al.*, 2022). More studies have shown that clinical symptoms of COVID-19 are seen to dramatically reduce after steam inhalation administration (Swain and Sahu, 2021). These results warrant that steam inhalation therapy (*Ukufutikila*) could have positive outcomes for COVID-19 management and control. Steam inhalation can potentially play a central role as a means for COVID-19 management in countries with huge hospital burdens. Not only in COVID-19 infections, but also in other respiratory infections in which it is commonly used as a home remedy. Besides, steam inhalation is an economically viable and convenient option (Chowdhury *et al.*, 2022).

5.4 Effect of *E. saligna* fresh leaves crude extract on SARS-CoV-2 infectivity of Vero T2 cells

Steam inhalation is usually practiced along with herbal remedies. The effect of steam inhalation in the presence and absence of herbal remedies on COVID-19 has not been extensively explored. Steam inhalation (*Ukufutikila*) is practiced in combination or presence of various species of *Eucalyptus* tree leaves. *Eucalyptus* species have been reported to be the most commonly used herbal medicine and especially the leaves being the most preferred plant parts for use (Chaachouay *et al.*, 2021; Villena-Tejada *et al.*, 2021). Mostly *eucalyptus* essential oils and those from other sources have been reported to be responsible for antiviral activities (Ma and Yao, 2020; Mieres-Castro *et al.*, 2021; Valussi *et al.*, 2021). Most of the studies utilize *Eucalyptus* tree dried leaves for all forms of extraction (Sadatrasul *et al.*, 2017; Maghsoodlou *et al.*, 2015). Several factors have been pointed out to influence the quality of herbal drugs including, the source of the raw material, crude botanical extracts, and methods of processing. These factors also play an important role in the therapeutic effects of crude plant material (Alamgir, 2017). In order to replicate what is practiced in steam inhalation as a home remedy and understand the possible role of *Eucalyptus* tree leaves, fresh *Eucalyptus* tree leaves were used to obtain a crude hydro-extract. Leaves of *Azadirachta indica*, *Eucalyptus globulus, Mangifera indica, Carica*

papaya, *Psidium guajava*, *Citrus reticulate*, and *Musa paradisiaca* have been steam-inhaled or orally taken in Africa and shown to offer relief from COVID-19 symptoms (Paudyal *et al.*, 2022). Furthermore, some medicinal herbal extracts have been assayed on plants such as *Cimicifuga rhizoma, Meliae cortex, Coptidis rhizoma, Phellodendron cortex* and *Sephorasubstrata radix*. These extracts were considered useful in the treatment of COVID-19 (Nur *et al.*, 2020). There is a good chance that steam inhalation infused with herbs would impede SARS-CoV-2 and ACE2 interaction (Elsebai and Albalawi, 2022). Therefore, this study endeavored to determine the effect of *E. saligna* fresh leaves crude extract on SARS-CoV-2 *in vitro* infectivity of Vero T2 cells.

This study established that there was no CPE observed in the Vero T2 cell cultures inoculated with SARS-CoV-2 material in the presence of 13% *E. saligna* fresh leaves crude extract. This strongly suggested that the crude extract had inhibitory effects on the infectivity of SARS-CoV-2 of the Vero T2 cells. Studies have shown, via *in silico* analyses, several components of *Eucalyptus* tree leaves' extracts or essential oil components' antiviral action. These components have been attributed to the disruption of secondary bonds involved in the interaction between SARS-CoV-2 spike glycoprotein and mammalian ACE-2 receptors. While others have been indicated as possible protease inhibitors (Yadalam *et al.*, 2021; Panikar *et al.*, 2021). In a similar manner, it is expected that this could explain the inability of SARS-CoV-2 to infect Vero T2 cells observed in this study. This is possibly due to failure to establish an interaction between SARS-CoV-2 spike protein and Vero T2 cells' ACE-2 receptors thereby preventing entry of SARS-CoV-2 in the Vero T2 cells. Essential oils have also been thought to interact with viral membrane lipids which may lead to compromising the integrity of the membrane and inhibit viral replication by binding the viral protease (Wani, Rouf *et al.*, 2021; Panikar *et al.*, 2021). However, it is also possible that other components of the crude extract could have also contributed to the failure of SARS-CoV-2 to infect the Vero T2 cells *in vitro*. This could be ascertained once the active ingredient(s) responsible for this inhibition is identified from the crude extract used in this study. The crude extract could have contained the reported essential oils and other volatile compounds present in the *Eucalyptus* tree fresh leaves as opposed to use of *Eucalyptus* tree dried leaves. With the limited time and resources for conducting this study, it was not possible to pursue this line of study.

5.5 Combined effect of heat inactivation and *E. saligna* fresh leaves crude extract on SARS-CoV-2 infectivity of Vero T2 cells

The study endeavored to determine the possible combined effect of heat inactivation and *E. saligna* fresh leaves crude extract on SARS-CoV-2 infectivity of Vero T2 cells. To do this, heat inactivated SARS-CoV-2 was inoculated in the presence of the *E. saligna* crude extract. This stemmed from the way steam inhalation is conducted in the presence of *Eucalyptus* tree fresh leaves as a home remedy.

According to the results of this study, there was 100% of assays with CPE observed in the positive control as compared to other cultures which were seen to have 0% of assays with CPE. This meant that after exposure to 13% *E. saligna* fresh leaves crude extract in media and heat, the SARS-CoV-2 was unable to infect the Vero T2 cells. This implied that the *E. saligna* fresh leaves crude extract and heat, had inhibitory effects on the infectivity of the SARS-CoV-2, respectively. Thus, this combined effect limited the *in vitro* infectivity of SARS-CoV-2 of the Vero T2 cells.

According to research, there is a correlation between CPE and detection of virus growth by RT-qPCR (Burton *et al.*, 2021). While other studies have used methods like 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or Immunofluorescence Assay among others to observe CPE (Sasaki *et al.*, 2021). This study relied on microscopy as this was sufficient to observe cell morphological changes like rounding, syncytia, detachment and plaque formation which were indicative of CPE. In this study, the CPE and virus growth was only observed in the positive control. The rest of the cultures exhibited no virus growth nor CPE. This is despite the presence of SARS-CoV-2 being detected by RT-qPCR in all the supernatants from the cultures. Additionally, there was no significant difference ($p = 0.771$) in the Ct-values between the positive control and the cultures exposed to *E. saligna* fresh leaves crude extract and heat. These results suggested that the SARS-CoV-2 present in the cultures was unable to replicate further after the *E. saligna* fresh leaves crude extract and heat exposures. But, the detected SARS-CoV-2 RNA could be attributed to that of the inoculated SARS-CoV-2 material. Lack of SARS-CoV-2 replication was possibly because the spike glycoprotein which plays a central role in infection was either inhibited by the crude extract or denatured by heat or affected by both treatments.

It has been noted that Ayurvedic practitioners use leaves and seeds of plants with steam inhalation against respiratory tract infections. Therefore, steam inhalation with aromatic oils of several plant parts would give positive outcomes in COVID-19 treatments (Chowdhury *et al.*, 2022). This study presents congruent results with this earlier study as it is seen (*in vitro*) that there is no viral growth nor CPE in cultures that were exposed to *E. saligna* fresh leaves crude extract and heat treated infectious material. With more research over time, steam inhalation therapy may result in a very positive COVID-19 management option and other associated diseases (Anam *et al.*, 2021). For example, in rural-urban populations of north India, home-based treatments like herbal steam inhalation were found to be the first line of action against COVID-19 and had significantly low COVID-19 incident rates (Radhika and Malik, 2021). The results of this study complement these earlier studies by providing a distinct experimental perspective on the combined action of heat and *E. saligna* fresh leaves crude extract on the *in vitro* infectivity of the SARS-CoV-2. The thermal inactivation of the SARS-CoV-2 using the entire temperature profile of steam inhalation for 20 minutes in combination with exposure to *E. saligna* fresh leaves crude extract show complete inhibition of SARS-CoV-2 *in vitro* infectivity.

This study has provided laboratory based evidence of the effectiveness of steam inhalation in the presence of *E. saligna* fresh leaves crude extract at inhibiting *in vitro* SARS-CoV-2 infectivity. The findings of this study indicated that heat treatment was effective in deactivation of the SARS-CoV-2 material and the *E. saligna* fresh leaves crude extract inhibited infectivity of the Vero T2 cells. Finally, the results showed that a combination of the two methods effectively prevented SARS-CoV-2's infectivity of Vero T2 cells. However, it was not possible to establish whether there was any synergist effect of the two treatments. Perhaps this should be the line of future related research as pointed out in section 6.2.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study draws the following conclusions;

1. The study confirmed that 20 minutes of steam inhalation per session attained a maximum average temperature of 94.7°C (at minute zero) which gradually reduced to an average of 54.4°C (at the twentieth minute).
2. The SARS-CoV-2 *in vitro* infectivity of the Vero T2 cells was evidently inhibited by *E. saligna* fresh leaves crude extract. Separately, heat treatment of SARS-CoV-2 resulted in the loss of the virus' infectivity.
3. Combined exposure of SARS-CoV-2 to *E. saligna* fresh leaves crude extract and the heat treatment of SARS-CoV-2 following the entire temperature profile attained in steam inhalation microenvironment inhibited SARS-CoV-2 *in vitro* infectivity of Vero T2 cells. However, the benefit of the combined use was not established.

6.2 Recommendations

The effects of different temperatures attained during steam inhalation with and without *E. saligna* fresh leaves crude extract on SARS-CoV-2 *in vitro* infectivity observed in this study, shed light on the possibility of steam inhalation being an efficient management alternative for COVID-19, especially in LMICS. This study recommends;

1. Clinical trials on positive COVID-19 asymptomatic patients and immediately exposed or before the onset of symptoms frontline workers should be conducted. This is to further have cognizance of the immediate effects of steam inhalation both in the presence and absence of *E. saligna* fresh leaves crude extract on COVID-19.
2. Accompanied by the safety explained from the results of this study, steam inhalation could be of benefit to frontline healthcare workers or asymptomatic COVID-19 patients in managing the disease hence may enhance ways of stopping the spread on the disease during at outbreak.
3. Future studies should be conducted to analyze the phytochemical composition of *E. saligna* fresh leaves crude extract as well as its effect on the host environment.

Further, purification and identification of active constituents responsible for inhibitory effects on SARS-CoV-2 infectivity of Vero T2 cells, of *E. saligna* fresh leaves crude hydro-distillation extract should be conducted since most of the reported studies used dried *Eucalyptus* spp. leaves samples.

4. Further studies are required to understand how other factors like pH may have interacted with temperature and the resulting effects on SARS-CoV-2 infectivity.

6.3 Study limitations

1. The conventional thermometers used in this study to determine temperature in the steam inhalation setup may have given less sensitive temperature readings. Use of thermocouple thermometers which are more sensitive is likely to give better temperature readings.
2. The bounds of this study did not endeavor to investigate the synergism of secondary metabolites or volatile components of *E. saligna* fresh leaves on the effects seen on SARS-CoV-2 *in vitro* infectivity.

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APPENDICES

Appendix A. Ethical Clearance from ERES Converge IRB

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30th May, 2022.

Ref. No. 2022-Mar-019

The Principal Investigator
Mr. Chisanga Chipanta
C/O The University of Zambia
School of Veterinary Medicine
Department of Disease control
P.O. Box 32379
Lusaka, Zambia

Dear. Mr. Chipanta

RE: PLAUSIBLE EFFECTS OF DIFFERENT TEMPERATURE ATTAINED DURING STREAM INHALATION WITH AND WITHOUT EUCALYPTUS GRANDIS GRANDIS LEAF EXTRACTS ON SARS-CoV-2 IN -VITRO INFECTIVITY.

Reference is made to your protocol submission. The IRB resolved to approve this study and your participation as Principal Investigator for a period of one year.

Review Type	**Fasttrack**	Approval No. **2022-Mar-019**
Approval and Expiry Date	Approval Date: 30th May,2022	Expiry Date: 29th May, 2023
Protocol Version and Date	Version - Nil.	29th May, 2023
Information Sheet, Consent Forms and Dates	• English.	29th May, 2023
Consent form ID and Date	Version - Nil	29th May, 2023
Recruitment Materials	Nil	29th May, 2023
Other Study Documents	Data Collection Sheet, Focus Group Discussion.	29th May, 2023
Number of participants approved for study	-	29th May, 2023

Specific conditions will apply to this approval. As Principal Investigator it is your responsibility to ensure that the contents of this letter are adhered to. If these are not adhered to, the approval may be suspended. Should the study be suspended, study sponsors and other regulatory authorities will be informed.

Conditions of Approval

- No participant may be involved in any study procedure prior to the study approval or after the expiration date.
- All unanticipated or Serious Adverse Events (SAEs) must be reported to the IRB within 5 days.
- All protocol modifications must be IRB approved prior to implementation unless they are intended to reduce risk (but must still be reported for approval). Modifications will include any change of investigator/s or site address.
- All protocol deviations must be reported to the IRB within 5 working days.
- All recruitment materials must be approved by the IRB prior to being used.
- Principal investigators are responsible for initiating Continuing Review proceedings. Documents must be received by the IRB at least 30 days before the expiry date. This is for the purpose of facilitating the review process. Any documents received less than 30 days before expiry will be labelled "late submissions" and will incur a penalty.
- Every 6 (six) months a progress report form supplied by ERES IRB must be filled in and submitted to us.
- A reprint of this letter shall be done at a fee.

Should you have any questions regarding anything indicated in this letter, please do not hesitate to get in touch with us at the above indicated address.

On behalf of ERES Converge IRB, we would like to wish you all the success as you carry out your study.

Yours faithfully,
ERES CONVERGE IRB

Dr. Jason Mwanza
Dip. Clin. Med. Sc., BA., M.Sc., PhD
CHAIRPERSON

Appendix B. Participant Information Sheet

Dear Participant,

Introduction:

My name is Chisanga Chipanta, I am pursuing my Master of Science Degree in One Health Laboratory Diagnostic Sciences from the University of Zambia. I intend to conduct a study on whether Steam Inhalation (Ukufutikila) can stop the virus that causes COVID-19 from infecting Humans. The study will be conducted at the University of Zambia clinic and the School of Veterinary Medicine.

Corona Virus Disease of 2019 (COVID-19) is an infection caused by Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) which is a highly transmissible emerging pathogen in humans. It has caused global public health emergencies including economic crises and millions of infections and thousands of deaths continue to be reported world over.

Purpose of the study:

The main aim of my research is to determine the effects of different temperatures attained during steam inhalation on the ability of the virus that causes COVID-19 to infect mammalian cells. Besides, I am conducting this study as partial fulfilment of the requirement to obtain a Master of Science Degree in One Health Laboratory Diagnostic Sciences from the University of Zambia.

Procedures:

Adult participants aged 18 years and above without underlying health issues will be recruited onto the study on a voluntary basis. The volunteers will undergo steam inhalation (Ukufutikila) at the University of Zambia Clinic under the supervision of the Medical Officer. Each steam inhalation session may last up to 20 minutes and will be repeated three times within 48 hours. A participant will be free to choose when to undergo steam inhalation. Once the temperatures of the steam in the steam inhalation process have been recorded, the participant ceases to take any further part in the study. The measured temperatures will be used in the experimental component of the study. This will involve optimising temperature effects on alkaline phosphatase activity and optimised temperatures will then be used to establish their effect on SARS-CoV-2 in-vitro infectivity of Vero cells.

Reason for invitation to participate in the research study:

This information will help us understand whether steam inhalation (Ukufutikila) is a useful tool in the management of COVID-19. The success of the study depends on measuring the temperatures which are reached during steam inhalation as it is done in homes. Therefore, your input is of critical importance to understand the experimental justification of steam inhalation as a COVID-19 management alternative.

Risks, Benefits, Discomforts of taking part in the study

Risks - There may be a risk of getting in direct contact with boiled water. However, the design of the steam inhalation stand will create a safe distance at least 25 cm between the surface of the boiled water and any part of your body. Steam inhalation will be carried out under close supervision of the Medical Officer and the Principal Investigator.

Benefits - COVID-19, being a new disease, it does not have specifically designed drug that are easily accessible. Therefore, validating the usefulness of steam inhalation in COVID-19 management will provide a possible opportunity of managing the disease and its spread at almost no cost.

Discomforts - Fatigue or stress may arise from undertaking the steam inhalation on three occasion. To avoid this, you are free to choose when to under the three sessions of steam inhalation although this has to be done within 48 hrs. Moreover the presence of the Medical Officer during the session will assure avoidance of any harmful effects these discomfort and the sessions can be stopped at any time

Privacy and Confidentiality:

Your personal information will not be shared with any third party or reported for the purposes of confidentiality. The findings of the experiments will be presented in an aggregated form, without revealing the identity of the participants.

Voluntary participation:

Your participation is strictly based on your willingness to volunteer and for this reason you are at free to withdraw from the study at any point. Please be informed that taking part in this study will attract no monetary gift or appeasement.

Access, Storage and Disposal

The temperature profiles obtained in the steam inhalation sessions will first be noted and recorded on hardcopy data sheets. The study will not request for any type of sample which will

be directly used in the analysis of the data. Afterwards, the temperature profiles will be stored on excel spreadsheets where they can be easily accessed for further data analyses.

Provision for standard of care.

Your will only be required to undergo steam inhalation sessions in this study. You will NOT be required to ingest or take any form of medication to participate in the study. The Medical Officer and principal investigate will be present during the session to ensure your safety and to take care of any eventualities that may arise.

Reimbursement to the participants

You will not incur any expenses in any form by participating in this study, therefore you will not be entitled to any reimbursement.

Contact Address

Should you need more information, please contact the under listed.

Chisanga Chipanta (Mr) University of Zambia, School of Veterinary Medicine, Department of Biomedical Sciences P.O. BOX 32379, Lusaka, Zambia. Mobile: +260971620618.

The Chairperson, ERES Converge, Plot No. 272, Cnr. Olive Tree Meanwood Road, Meanwood Ibex, Lusaka, Zambia. Tel: +260955155633

Appendix C. Informed Consent

STUDY PLAUSIBLE EFFECTS OF DIFFERENT TEMPERATURES ATTAINED DURING STEAM INHALATION WITH AND WITHOUT *EUCALYPTUS GRANDIS* LEAF EXTRACTS ON SARS-CoV-2 INFECTIVITY

TO The Participant

We are interested in finding out whether the temperature of steam in Ukufutikila also called Steam Inhalation can stop the virus that causes COVID-19 from infecting Humans. We are therefore requesting you to participate in the study by undergoing steam inhalation for three times with each session lasting not more than 20 minutes. You will be allowed to choose when to undergo steam inhalation within a period of 48 hours at the University of Zambia Clinic.

I confirm that I have read and understand the Participant Information Sheet	Yes	No
I have had the opportunity to ask questions and had them answered	Yes	No
I understand that all personal information will remain confidential and that all efforts will be made to ensure I cannot be identified (except as might be required by law)	Yes	No
I agree that data gathered in this study may be stored anonymously and securely, and may be used for future research	Yes	No
I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason.	Yes	No
I agree to take part in this study	Yes	No

By signing your name at the bottom means that you agree to be in this study. You will be given a copy of this form after you have signed it.

I....., consent to participate in this study.

Participant's signature..... Date.....

Witness's signature..... Date.....

Name of investigator.....Investigator's signature.....

Date.....

Persons of contact in case of any problems.

Chisanga Chipanta (Mr) University of Zambia, School of Veterinary Medicine, Department of Biomedical Sciences P.O. BOX 32379, Lusaka, Zambia. Mobile: +260971620618.

The Chairperson, ERES Converge, Plot No. 272, Cnr. Olive Tree Meanwood Road, Meanwood Ibex, Lusaka, Zambia. Tel: +260955155633.

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Appendix D. Health and Procedural Checklist

Please indicate **YES** or **NO** by crossing.

	SYMPTOMS.	RESPONSE	
1.	Have you undertaken a steam inhalation session before?	**YES**	**NO**
2.	Do you have fever?	**YES**	**NO**
3.	Are you experiencing body aches?	**YES**	**NO**
4.	Are you having difficulty breathing?	**YES**	**NO**
5.	Do you have a headache?	**YES**	**NO**
6.	Do you have cough?	**YES**	**NO**
7.	Are you experiencing severe chest pain?	**YES**	**NO**
8.	Are you experiencing shortness of breath?	**YES**	**NO**
9.	Are you experiencing chills?	**YES**	**NO**
10.	Are you experiencing loss of sense of smell or taste?	**YES**	**NO**
11.	Do you have sore throat?	**YES**	**NO**
12.	Are you experiencing stuffy nose?	**YES**	**NO**
13.	Are you experiencing nausea, vomiting or diarrhoea?	**YES**	**NO**
14.	Are you experiencing any muscle or joint ache?	**YES**	**NO**
15.	Are you feeling unwell, Fatigue or severe exhaustion?	**YES**	**NO**
16.	Do you have any long standing illness for example Diabetes, HIV/AIDS, Asthma, Hypertension...? Indicate the illness	**YES**	**NO**

*Participant's Name:*_____ *Sign*_____

*Date*_____

Appendix E. Permission Letter from UNZA Clinic

THE UNIVERSITY OF ZAMBIA
HEALTH SERVICES

INTERNAL MEMORANDUM

TO	:	Head, Department of Biomedical Sciences
FROM	:	Medical Officer
DATE	:	12th May 2022
SUBJECT	:	**REQUEST TO CONDUCT STEAM INHALATION AT UNZA CLINIC AND SCREEN POTENTIAL VOLUNTEERS**

I draw your attention to the above subject matter and your memorandum dated 10th May 2022.

I write to inform you that UNZA Clinic has no objection to facilitate the process of screening potential participants and monitoring of the steam inhalation process in support of the project.

Please do not hesitate to contact my office should you require additional information.

C. S. LWATULA

Dr Clementina Lwatula
MEDICAL OFFICER

APPROVED
30 MAY 2022
ERES CONVERGE
P/BAG 125, LUSAKA.

Appendix F. Permission Letter from NHRA

NATIONAL HEALTH RESEARCH AUTHORITY
Paediatric Centre of Excellence, University Teaching Hospital, P.O. Box 30075, LUSAKA
Chalala Office Lot No. 18961/M, Off Kasama Road, P.O. Box 30075, LUSAKA
Tell: +260211 250309 | Email: znhrasec@nhra.org.zm | www.nhra.org.zm

Ref No: NHRA0000024/01/06/2022 **Date: 1st June, 2022**

The Principal Investigator,
Chisanga Chipanta
UNZA VET,
Lusaka, Zambia

Dear Dr Chipanta,

Re: Request for Authority to Conduct Research

The National Health Research Authority is in receipt of your request for ethical clearance and authority to conduct research titled **"Plausible Effects of Different Temperatures Attained during Steam Inhalation with and without Eucalyptus grandis Leaf Extracts on SARS-CoV-2 in-vitro Infectivity."**

I wish to inform you that following submission of your request to the Authority, our review of the same and in view of the ethical clearance, this study has been approved on condition that:

1. The relevant Provincial and District Medical Officers where the study is being conducted are fully appraised;
2. Progress updates are provided to NHRA quarterly from the date of commencement of the study;
3. The final study report is cleared by the NHRA before any publication or dissemination within or outside the country;
4. After clearance for publication or dissemination by the NHRA, the final study report is shared with all relevant Provincial and District Directors of Health where the study was being conducted, University leadership, and all key respondents.

Yours sincerely,

Prof. Victor Chalwe,
Acting Director
National Health Research Authority