IN VITRO ANTICANCER AND ANTI-INFLAMMATORY POTENTIAL OF TIAN IMMUNE BOOSTER

A MINI THESIS SUBMITTED IN PARTIAL FULFILMENT

OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE (INDUSTRIAL BIOCHEMISTRY)

OF

THE UNIVERSITY OF NAMIBIA

BY

MOSES KAUFIWENI HAILUME

201408366

APRIL 2021

SUPERVISOR: PROF PETRINA KAPEWANGOLO (Department of Chemistry and Biochemistry, University of Namibia)

LIST OF CONFERENCE PROCEEDINGS

M. Hailume, P. Kapewangolo, *in vitro* anticancer potential of Tian Immune Booster: investigating against cervical and skin cancer cell models. National Students' Research Symposium. 2nd-3rd October 2019. (Presentation).

ABSTRACT

Tian Immune Booster (TIB) is a Chinese herbal product made up of more than 30 different Chinese herbs and formulated in sugar-coated tablets. TIB is currently approved for use in China, Kenya and Zambia to manage liver and kidney diseases as well as HIV and cervical cancer. The use of TIB to manage diseases is currently based on traditional knowledge and clinical evidence, but scientific validation for this use is not available. Chronic inflammation is one of the contributing factors to the development of different diseases. The current study was aimed at investigating the *in vitro* anticancer and anti-inflammatory potential of TIB extracts. Anticancer and anti-inflammatory data from the present study may provide more insight into biological activities of TIB in terms of the potential to treat cancer and protection against inflammation respectively. The anticancer effects of TIB towards human cervical cancer (HeLa) and human malignant melanoma cell lines was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at extract concentrations between 1.56 and 100 µg/mL. The albumin denaturation assay was used to evaluate the anti-inflammatory potential of TIB at concentrations between 3.12 and 200 µg/mL. The anticancer and anti-inflammatory potential of TIB was tested for single and combined extracts. No anticancer activity was observed with TIB towards HeLa and melanoma cells, with IC_{50} values ≥ 100 µg/mL for all extracts. TIB exhibited good anti-inflammatory activity, with IC_{50} values ranging from 14.53 ± 0.87 to 163.84 ± 10.59 µg/mL for single extracts and from 12.30 ± 0.29 to 44.85 ± 2.77 µg/mL for combined extracts. Minimal anticancer activity may be an indication that TIB does not directly kill cancer cells. Good anti-inflammatory activity revealed by this study support the traditional use of TIB. In future, *in vivo* anticancer and anti-inflammatory studies on TIB may be considered.

Keywords: TIB, MTT, Anticancer, Albumin denaturation, Anti-inflammatory

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND ACRONYMS

AI:	Activity index
AIDS:	Acquired Immune Deficiency Syndrome
ART:	Antiretroviral treatment
ATP:	Adenoside triphosphate
BSA:	Bovine serum albumin
BSL:	Biosafety Level
IC_{50}:	50% cytotoxic concentration
CHMs:	Chinese Herbal Medicines
COX:	Cyclooxygenase
CO_2:	Carbon dioxide
DAMPS:	Damage-associated molecular pattern molecules
DMEM:	Dulbecco's Modified Eagle's Medium
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
FBS:	Fetal Bovine Serum
GZJSYT:	Gui-Zhi-Jia-Shao-Yao-Tang

HIV:	Human Immunodeficiency Virus
HRBC:	Human bed blood cells
IC_{50}:	50% inhibitory concentration
LC-MS:	Liquid chromatography-mass spectroscopy
LPS:	Lipopolysaccharides
LTs:	Leukotrienes
LOX:	Lipoxygenase
MAPK:	Mitogen-activated protein kinase
MEM:	Minimum Essential Medium
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTS:	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NADH:	Nicotinamide Adenine Dinucleotide Hydride
NF-κB:	Nuclear factor-kappa B
NMR:	Nuclear Magnetic Resonance
NO:	Nitric oxide
NSAIDS:	Non-steroidal Anti-inflammatory Drugs

NSFAF:	Namibia Students Financial Assistance Fund
OA:	Osteoarthritis
PBS:	Phosphate-buffered saline
PAMPS:	poison-associated molecular pattern molecules
PGE2:	Prostaglandin E2
PGs:	Prostaglandins
PI:	Propidium iodide
QF:	Qingfei
QDS:	Qingdaisan
RANTES:	Regulated upon activation, normal T-cell expressed and secreted
RNA:	Ribonucleic acid
RNS:	Reactive Nitrogen Species
ROS:	Reactive Oxygen Species
SEM:	Standard error of mean
SI:	Selectivity Index
STIs:	Sexually transmitted infections
SRB:	Sulphorhodamine B

TB:	Tuberculosis
TCM:	Traditional Chinese Medicine
TIB:	Tian Immune Booster
TIB comb.	Tian Immune Booster combination
TNF-α:	Tumor Necrosis Factor α
UNAM:	University of Namibia
WHO:	World Health Organization
WST-1:	Water Soluble Tetrazolium 1
XFZY:	Xuefu Zhuyu decoction
XTT:	2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl) -2H-tetrazolium

ACKNOWLEDGEMENTS

I would like to acknowledge the following individuals and institutions for their contribution to this research. First and foremost, I thank The Lord for everything is possible through him. Secondly, I would like to express my gratitude to the Namibia Students Financial Assistance Fund (NSFAF) for providing the funds required to carry out this research project. In addition, I thank Prof Petrina Kapewangolo for the best supervision, encouragement and training. I am grateful to Prof. Tian Shengxun who allowed this study to be carried out on his products, TIB. I appreciate the UNAM School of Medicine staff for availing laboratory space where cell culture experiments were carried out. The contribution of UNAM Main Campus staff members to this study is also highly appreciated. My final appreciation goes to my family members and friends for their social and emotional support during the period of this study.

DEDICATION

I would like to dedicate this research work to the TIB Research Centre in Kenya where the TIB used in this study is developed and manufactured. I further dedicate this study to all HIV and cancer sufferers around the world as well as various institutions that participate in HIV and cancer research as well as those carrying out various practices to assist HIV and cancer sufferers worldwide. Lastly, I would like to dedicate this research to different herbal medicine practitioners that employ medicinal plant products in the fight against otherwise incurable and devastating ailments.

DECLARATIONS

I, Moses Kaufiweni Hailume, hereby declare that this study is my own work and is a true reflection of my research, and that this work, or any part thereof has not been submitted for a degree at any other institution.

…… ……..…… ……

Name of Student Signature Date

1. INTRODUCTION

1.1 Background of the study

Tian Immune Booster (TIB) is a Chinese herbal mixture made up of more than 30 different herbs and formulated in sugar coated-tablets for administration [1]. TIB has been approved for use in China, Kenya and Zambia by the respective authorities (governments) and is popularly administered in these countries for the treatment of liver and kidney diseases as well as for the management of HIV and cervical cancer [1,2]. *In vitro* evidence of the anti-HIV activity of TIB exists [3] but there are no scientific reports evaluating its anticancer or anti-inflammatory activities. Data from the present study may help reveal the biological activities of TIB in terms of cellular toxicity and protection against inflammation.

The traditional use of medicinal plant products to manage diseases is more common in developing countries as compared to the developed world [4,5]. About 25% of all approved drugs are derived from plants [6–9]. Plants used in traditional medicine display few or sometimes no side effects and this encourages researchers to carry out more research focusing on the biological activity of medicinal plants [10]. Collecting plant samples is a very low intensity labor, saving time and energy in the process [36]. With plants being environmentally friendly and renewable sources of active compounds, using plant-derived products for therapeutic purposes provides an additional benefit of availability and sustainability [36]. Plants that are already used for the management of one or more diseases usually yield promising results when tested for the potential against other diseases and alleviate symptoms from different diseases [4].

The traditional use of medicinal plant products to manage diseases has shown promising results in different patients whereby various studies have indicated that phytochemicals produced by plants are responsible for the biological activity [11,12]. Phytochemicals target a variety of processes, causing diverse alterations that may lead to successful treatment of diseases [12,13]. The use of traditional medicine as alternative therapy has been on the increase, however safety and efficacy associated with the use of herbal remedies can only be assessed through scientific investigation and experimentation [4,14,15]. Biological activities of medicinal plants constantly studied include the potential to manage or treat different diseases through anti-inflammatory mechanisms [16,17].

In the event of tissue injury, infection by pathogens or exposure to toxic substances, the body responds by mediating immunity mechanisms to assist with tissue repair and pathogen destruction [18]. This physiological response is called inflammation [19,20]. Inflammation is associated with redness, swelling, pain, abnormal tissue functionality and heat [21,22]. Despite the primary purpose and outcome of acute inflammation being to heal the body, tissue damage may occur during lengthy inflammation, also called chronic inflammation [18,19].

One of the consequences of chronic inflammation is protein denaturation, whereby proteins lose normal functionality [23]. Denatured proteins contribute to the rise in immune cells at the site of inflammation and subsequently, the immune cells contribute to even more inflammation or chronic inflammation [23,24]. It is therefore reasonable to consider substances that inhibit denaturation of proteins as good anti-inflammatory agents. Substances that inhibit protein denaturation bind to the proteins at regions that

induce protein denaturation and at receptor motifs responsible for inducing inflammation, in turn preventing inflammation [25,26].

Lysosomal enzymes are usually released during inflammation, causing macromolecular damage and the peroxidation of cellular lipids [19]. Tissue damage that occurs during chronic inflammation may lead to other complications including cancer, cardiovascular diseases and rheumatoid arthritis [17,19,27]. Due to additional damage to the body that may arise as a result of chronic inflammation, it is essential to develop medication that alleviates or eradicates the unfavorable consequences of inflammation.

Drugs widely used to treat inflammation belong to the category of non-steroidal anti-inflammatory drugs (NSAIDs) [28]. The ability to inhibit protein denaturation is a collective feature of anti-inflammatory drugs including acetylsalicylic acid, ibufenac [22], ibuprofen and dichlofenac [29]. In spite of the promising anti-inflammatory activity displayed by NSAIDs, the use of such drugs may result in unfavorable side effects including kidney damage, gastric ulcers, liver damage and heart problems [18,22,23,30]. The occurrence of side effects reported with the use of NSAIDs encourages studies focusing on developing novel anti-inflammatory agents with improved safety in an effort to tackle contributing effects of inflammation to other diseases such as cancer [6]. *Cassia auriculata*, *Tamarindus indica* and *Anvillea radiata* are among a long list of medicinal plants traditionally used to manage inflammation and were found to possess anti-inflammatory activity through scientific studies [31–33]. Cyclooxygenase (COX)-inhibiting plant extracts have been previously obtained from different plants including *Harpephyllum caffrum*, *Merwilla plumbea* and *Cola*

greenwayi [34]. The list of conditions that are traditionally managed with medicinal plants goes beyond inflammation.

Cancer is regarded the second leading cause of death after cardiovascular disease [1]. About 1 in every 6 deaths of people is caused by cancer worldwide [35]. The World Health Organization (WHO) has reported a total of 1 055 172 cancer cases in Africa during 2018, whereby 693 487 deaths caused by cancer have been reported [36]. In Namibia, a total of 700 mortalities resulting from cancer have been reported during 2014 with females being the most affected gender [37]. A total of 400 mortalities of females were reported compared to 300 mortalities in males [37]. The majority of deaths in Namibian females in 2014 were a result of breast cancer, constituting 22.3%, to which cervical cancer was second with 16.2% [37]. From 2004 to 2012, there has been a notable decrease in mortality cases due to cancer in Namibia indicating improvements in treatment [37]. However, with increasing choices of lifestyles among individuals as well as the consistently increasing world population, cancer statistics are expected to exponentially increase, unless alternative and more effective treatment options are developed [38]. Fortunately, traditional Chinese medicine (TCM), which is under constant investigation for the potential to manage different diseases may be developed into appropriate adjunctive therapy to improve and prolong the lives of patients suffering from cancer [39].

Mortalities resulting from different diseases are highly influenced by the effectiveness of drugs that are presently administered to patients [1]. Most anticancer drugs currently used have limited effectiveness and the use of such drugs may result in side effects such as heart diseases and kidney failure [1]. In the treatment of cancer, poor selectivity

causes anticancer drugs to target non-cancerous cells during treatment, leading to toxic side effects [40]. Elevated costs associated with drug treatment by conventional means reduces the number of patients being actively involved and successfully completing such treatments [41]. The present study was aimed to investigate the *in vitro* anticancer alongside the *in vitro* anti-inflammatory potential of TIB extracts.

1.2 Statement of the problem

TIB is presently administered to patients based on traditional knowledge. There are currently no literature reports on the anticancer effect of TIB. The potential of TIB as an anti-inflammatory agent has also not been explored to date which could be a contributing factor of alleviating symptoms experienced by TIB patients.

1.3 Objectives of the study

The objectives of this study were to:

a) investigate the *in vitro* anticancer potential of single and combined TIB extracts against HeLa and Melanoma cell lines,
b) evaluate the anti-inflammatory potential of single and combined TIB extracts using the albumin denaturation assay

1.4 Significance of the study

The findings of this study may provide *in vitro* evidence supporting the traditional use of TIB. Anticancer data from the present study will help reveal possible cellular toxicity from TIB that users may currently not be aware of. Furthermore, the anti-inflammatory data may provide first-hand information on the interactions of TIB with proteins that could lead to effective treatment of diseases linked to inflammation, including cancer. The anti-inflammatory potential and minimal anticancer effect demonstrated by TIB in

this study could be attributed to its popular use in managing various ailments. The findings of this study will also create more awareness for TIB to be further investigated *in vivo* and in clinical settings.

1.5 Limitations of the study

There are many different types of commercially available cell line models that could have been explored, but have not been screened in this study. Due to limited resources, non-cancerous cell lines were not included in the study to represent cytotoxicity of TIB in healthy cells. In addition, a wide variety of *in vitro* assays such as the sulphorhodamine B (SRB) [42], resazurin [43] as well as membrane stabilization and heat-induced hemolysis [44] exist, that are popularly used for assessment of the anticancer and anti-inflammatory potential of substances respectively, but could not all be tested in the present study due to limited resources. Various positive controls for anti-inflammatory assay including Disprin and Anadin, both containing acetylsalicylic acid as the active ingredient, were tested but did not work. Lastly, TIB samples were not used as whole entities but were extracted with methanol/dichloromethane solvent system. Therefore, the study is only a representation of TIB constituents soluble in the mentioned solvent system.

1.6 Delimitations of the study

This study focused on investigating the anticancer of TIB on two cancer cell lines namely; cervical (HeLa) and skin (Melanoma) cancer cell models. The anti-inflammatory activity of TIB was studied using the albumin denaturation assay. The anti-inflammatory activity of TIB was tested with untreated albumin acting as the negative control.

2. LITERATURE REVIEW

2.1 Medicinal plants administered in traditional settings

2.1.1 Medicinal plants in disease management

In search of more effective and less toxic therapeutic agents, researchers are beginning to direct their focus on the potential of natural products to manage diseases and associated symptoms [15,45,46]. Natural products derived from plants [47], fungi [48,49] or marine organisms [45] show promising potential as health-promoting agents. Plants constitute a large fraction of sources of various therapeutic substances and promising findings exist on the potential of plant-derived substances to manage diseases [46,50]. Studies focusing on the potential of plant extracts as therapeutic substances observed bioactivity that is attributed to the presence of phytochemicals such as alkaloids, flavonoids and tannins produced by plants [46,51]. The need for further investigation into application of herbal medicine is evident in specific cases such as cancer, where current treatment options have limited effectiveness and may be associated with cellular toxicity [52].

Traditionally, plants have been used to manage various ailments and have been found to be effective against a wide variety of ailments [49]. The application of traditional medicine by employing plant extracts and active constituents of extracts from plants contributes up to 80% to the world's primary health care [53]. Patients in different parts of the world may receive traditional medication from traditional medical practitioners with the aim of curing or delaying the effects of diseases or to alleviate symptoms resulting from side effects of conventional treatments being administered to patients [54]. However, there is a need for detailed scientific assessment for any possible

resulting side effects and the possible source of side effects as well as scientific evaluation of the efficacy of plant-derived compounds. At the Arokhayasala Foundationat Wat Khampramong in Thailand, a number of cancer patients are administered with a formula made up of 11 herbs in combination with other therapies such as meditation and prayer [54]. Promising results on the herbal formula have been observed, however in the events of any side effects from a treatment such as this, it is impossible to clinically single out a specific source of any reaction to the treatment as the effect may be a result of interactions between two or more methods or plant constituents [55,56].

Herbal medicine have been widely applied for protective and palliative purposes, in the management of cancer, a disease that poses a global burden [55]. The use of herbal products largely in combination with conventional treatments of chemotherapy, radiotherapy and surgery is continuously gaining favor over the conventional treatment options used individually [56].

Current scientific activities are guided by reported traditional use of plants to determine the components as well as mechanisms responsible for the effects observed in the clinical environment [46]. Where good biological activity is observed from tests on plant extracts, plants can be specifically screened for active compounds with the goal of determining their exact modes of action [57]. Screening of plant products for bioactivity is carried out by employing both *in vivo* and *in vitro* assays whereby herbs are tested in isolation as well as in combination with one another [54]. Ongoing research on anticancer activity of herbal products has led to various herbal extracts found to possess biological activities ranging from the ability to induce apoptosis to the ability to inhibit

cancer cell replication by upregulation of tumor suppressor genes and down regulation of oncogenes [58]. The shift in focus of researchers to explore herbal anticancer is motivated by the poor success rate of conventional therapy and by the promising potential displayed by medicinal plants [59].

2.1.2 Selected medicinal plants of Southern Africa

Plant parts such as leaves, barks, roots and fruits have long been used in Africa to manage diseases by chewing, mixing, boiling and administering to patients [57,60,61]. Sub-Sahara Africa is rich in a wide array of plants that can be used in medicinal applications [62]. In addition to the main preliminary uses of herbs as food and natural remedies, herbs derived from different parts of the world including Africa are constantly being transformed into pharmaceuticals [63]. Plant products used for medicinal purposes mostly as immune boosters are well packaged and sold around Southern Africa in spite of the products having not been scientifically tested for efficacy and safety, only traditional uses guide the commercialization and use of the plant products [64]. Selected medicinal plants used in Southern Africa to manage different diseases are presented in Table 1.

Table 1. Selected medicinal plants from southern Africa used to manage various diseases.

Plant species	Traditional medicinal applications	References
Centella asiatica	Cancer, wounds, acne, allergies, leprosy	[62]
Elytropappus rhinocerotus	Stomach cancer, ulcers, appetite stimulant	[62]
Sutherlandia frutescens	Cancer tonic, skin and eye disorders, diabetes	[62]
Vernonia	HIV	[65]

amygdalina		
Hypoxis hemerocallidea	HIV, immunity booster	[65,66]
Vernonia amygdalina	HIV	[65]
Justicia flava	Cough, paralysis and epilepsy	[67]
Ximenia caffra	Sexually transmitted infections (STIs), stomach-ache and fever	[68]
Kigelia africana	Syphilis, cancer and fungal infections	[67]
Tabernaemontana ventricosa	Wounds, sore eyes and wounds	[69]
Aloe vera	Wounds	[70]
Marrubium vulgare	Wounds	[70]
Elaeis guneensis	Wounds	[70]
Rapanea melanophloeos	Influenza	[69]
Pittosporum viridiflorum	Influenza	[69]
Bridelia mollis	Malaria, cough and STIs	[71]
Psidium guavaja	Cough, flu and fever	[72]
Terminalia prunioides	STIs, abdominal pain and backache	[71]
Agave sisalana	Wounds, diarrhea, dysentery, jaundice, flatulence, indigestion and constipation	[73]
Centella asiatica	Cancer, wounds, fever, leprosy and syphilis	[73]
Bulbine latifolia	Contraception	[74]
Centaurea benedicta	Contraception	[74]
Warburgia ugandensis	Malaria	[75]
Acacia xanthophloea	Malaria	[75]
Agapanthus africanus	Cancer	[76]

Euphorbia ingens	Breast cancer	[76]
Acacia burkei	Ringworm infections	[77]
Crinum bulbispermum	Skin infections	[77]
Acacia mearnsii	Flu and influenza	[78]
Achyranthes aspera	Bronchitis and colds	[78]
Alepidea amatymbica	Respiratory infections and abdominal disorders	[34]

Medicinal plants found in Southern African continue to be useful in communities as the need for sanitation continues and infections are constantly contracted through unclean food and water [75]. Scientific studies are constantly providing explanations for the part played of African medicinal plants in the healing process through the use of plants to treat a wide array of microbial infections [66].

Plants have long been utilized in Southern Africa to treat wounds in traditional settings, owing the healing benefits to a wide range of constituents of medicinal plants used in healing applications [68]. Plants used to treat wounds include *Aloe vera, Marrubium vulgare* and *Elaeis guneensis* [70]. The progress in management of wounds using medicinal plants depends on the ability of plant constituents to interact with several biological processes including blood coagulation, inflammation, wound contraction and collagen accumulation at the wound [68]. Studies have shown that, plants and plant products with wound healing properties have in common, antioxidant properties, keeping reactive oxygen species (ROS) and reactive nitrogen species (RNS) under safe physiological levels during the healing process [61]. Oxidative stress delays the healing

process as it promotes constant cell death and regeneration, therefore medicinal plants widely used in Southern Africa that display antioxidant potential are promising candidates for treatment of wounds, among other diseases [61].

Human and animal infection by influenza virus is another Southern African burden that may require intervention by application to herbal remedies already at disposal of the African continent [69]. Extracts from various South African medicinal plants including *Rapanea melanophloeos* and *Pittosporum viridiflorum* have been studied and demonstrated effectiveness against influenza virus A [69].

2.1.3 Traditional Chinese Medicine

Initially, conventional cancer therapy had dominated western countries while TCM was used more in eastern countries with more developments in China [79,80]. TCM gained popularity and is applied to other countries resulting in convergence between the two groups of treatments and hence are sometimes used in combination with one another [80]. After several successes in the communities and research brought about by TCM, including the discovery and approval of artemisinin for the management of malaria, research focused more on the application of TCM in the healthcare sector [81].

In TCM, herbs are largely employed to target more than one ailment, taking advantage of the large array of active principles present in herbs [81]. Although some herbs are used as a single plant sample, some are formulated into decoctions with multiple applications in healthcare such as in treatment of osteoarthritis by anti-inflammatory and anticancer mechanisms among other targets [82]. In addition to the safety of TCM products, the ability of TCM to enhance immunity, leading to healing and improved overall health, presents stimulation into research directed at healthcare by TCM thereof

[83]. TCM products exhibit anticancer activities via different pathways including the regulation of apoptosis, proliferation and metastasis in the direction if cancer cell destruction [84]. Artemisinin discovery rooted from the traditional application of *Arteimisia annua*-containing traditional remedy that was used by the Chinese in ancient times as a successful cure for malaria [85].

Despite the widespread use of the traditional approach to disease management using TCM, analysis and further development of TCM products may contribute largely to highly effective and safe western treatments [85]. Hence collaboration between traditional medicinal practitioners and western medicinal doctors may be the best approach to an efficient therapeutic future. This may help in curing diseases that are currently without cure. Osteoarthritis (OA) is one such disease without cure, but TCM products have shown promising activity against OA [82]. This result from, among others, the anti-inflammatory potential and the ability of herbal remedies to cause changes in bone structure, leading to strengthening bones and healing of the OA [82]. Furthermore, TCM products have the potential to prevent or treat inflammation as a result of the contribution by TCM constituents that may take part in the regulation of both pro-inflammatory and anti-inflammatory mediators [86].

Several TCM products that are made up of a combination of extracts exist and have been evaluated for biological activities, including anticancer and anti-inflammatory. PC-SPES is an example of such herbal formulae containing eight different herbs, that was developed for healthcare applications in 1996, more specifically for prostate cancer management [87]. This product was however removed from the market in 2000 due to reported side effects in patients using the product as well as the presence of toxic

compounds such as warfarin that was revealed through research [87]. Despite the general trend of low toxicity observed among various herbal medicines, there is still need to evaluate possible toxicity of plant products as some may still contain cytotoxic constituents and lead to unfavorable effects [88]. Several TCM products have been highlighted to cause nephrotoxicity, which could be attributed to specific nephrotoxic constituents of the TCM, contamination, processing and storage errors and interaction between co-administered medication [89]. It is therefore of paramount importance to note that after pharmacological activity has been observed with a specific product, side effects are essential to investigate. In case of TCM products without side effects, it is of great advantage to get the products developed further for clinical use due to the promising benefits.

Another TCM product made of a combination of herbs is Qingdaisan, also known as Formulated Indigo Naturalis powder or QDS used to manage a wide array of ailments including sores and ulcers [90]. QDS is a formula of Indigo naturalis and three herbal plants namely; *Coptis*, *Phellodendron*, *Mentha*, *Platycodon* and *Acacia*, all mixed in equal ratio by mass [90]. Indigo naturalis, a herbal drug used to manage psoriasis and with established activity against leukemia, is made by fermentation of five plant species extracts from *Baphicacanthus cusia*, *Polygonum tinctorium*, *Indigofera tinctorial*, *Isatis indigotica* and *Isatis tinctorial* containing simple sugars, starch and inorganic constituents [91]. ODS displays impressive *in vivo* anti-inflammatory activity against both chronic and acute inflammation alongside dose-dependent anti-ulcerogenic activity [91]. TCM products used for different medicinal applications are listed in Table 2.

Table 2. Selected TCM products made of combinations of herbs used for the management of a wide variety of diseases

TCM product	Constituents	Traditional uses	References
Qingdaisan	Indigo naturalis, *Coptis*, *Phellodendron*, *Mentha*, *Platycodon* and *Acacia*	Sores and ulcers	[90]
Indigo Naturalis	*Baphicacanthus cusia*, *Polygonum tinctorium*, *Indigofera tinctorial*, *Isatis indigotica* and *Isatis tinctorial*	Psoriasis	[91]
Xiang-Sha-Liu-Jun-Zi-Tang	*Panax ginseng*, *Atractylodes macrocephala*, *Wolfiporia extensa*, *Glycyrrhiza uralensis*, *Citrus reticulata*, *Pinellia ternate*, *Amomum villosum* and *Vladimiria souliei*	Lung cancer	[92]
Ma xing shi gan decoction	*Ephedra sinensis*, *Semen armeniacae amarum*, *Glycyrrhiza* and *Gypsum fibrosum*	Asthma and cough	[93]
Yin-Chen-Hao-Tang	*Artemisia annua*, *Gardenia jasminoides* and *Rheum palmatum*	Hepatic injury syndrome	[94]
Bushen Qinggan Formula	*Astragali mongholici*, *Rhizoma coptidis*,	Hypertension	[95]

	Pollen typhae, Rhizoma alismatis, and *Artemisiae capillaries*		
Liu-Wei-Di-Huang Wan	*Rehmannia glutinosa, Cornus officinalis,* Common Yam Rhizome, *Alisma orientalis,* Tree Peony Bark, *and Poria cocos*	Alzheimer's disease, hypertension, osteoporosis and diabetes	[96]

2.2 Tian Immune Booster

TIB is Chinese herbal product that is administered to patients in the form of tablets for management of liver and kidney diseases as well as against of HIV and cervical cancer [2]. Natural products have been referred to as being capable of suppressing the HIV virus replication as well as reactivate latent virus and expose those to the destruction by the immune system or ART if used in combination [97]. A Chinese patient was reported to have been cured of squamous cell carcinoma in 2019 upon TIB administration after diagnosis in August 2018 [98]. A previous study on TIB investigated its anti-HIV potential where it was reported to inhibit the virus up to 100% [3].

2.3 Inflammation

Any form of tissue damage ranging from a cut to bacterial infection, viral infection, radiation or damage from any other form of physical or chemical means triggers a non-specific response in an effort to counteract the destructive effect. This response is called inflammation [16,99]. Inflammation constitutes a complex defensive mechanism that undertakes a series of steps and stages that may or may not be successful in eliminating

the root cause of tissue damage alongside the subsequent effects (Fig. 1) [100,101]. When healthy cells interact with pro-inflammatory agents called stressors, the cells are signaled to release different inflammatory mediators depending on the type of stressor involved [101]. Most commonly encountered pro-inflammatory mediators are damage-associated molecular pattern molecules (DAMPs) and pathogen-associated molecular pattern molecules (PAMPs) released by healthy cells as a result of exposure to toxic chemicals and pathogens respectively [101]. These molecules are subsequently involved in the initiation of inflammatory pathways such as DNA or RNA activation as well as protein and bacteria-derived lipopolysaccharide modification [101].

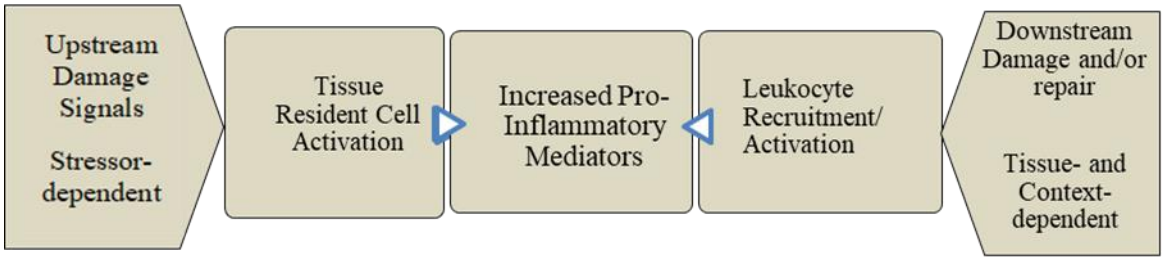


Figure 1. A summary of series of steps taken through the progression into inflammation. The consequence could be of healing or damaging impact [101].

In event of invasion by pathogenic organisms, macrophages and mast cells, together with other inflammatory cells are activated with a single purpose of cytokine and chemokine production [102]. Cytokines and chemokines, working together with any present pathogenic antigens initiate the migration of circulating leukocytes, which are transported to the site of damage with the help of vesicles, ready to eliminate any pathogenic substances [102]. Sequential and parallel pathways are initiated employing selectins, integrins, neutrophils and monocytes that act in the defense and healing of any damaged cells and tissues [101,103,104]. Glycoconjugates on leucocytes reversibly

interact with selectin, leading to leucocyte tethering as well as rolling of lysosomes towards the cite of inflammation for the leukocyte recruitment in the inflammation process [103,105]. In addition to leukocyte rolling, adhesion, migration through and crawling within blood vessel walls are processes that play a role in the introduction and recruitment of leukocytes at the site of inflammation [106].

Lipoxygenase (LOX) and COX pathways contribute greatly to the inflammation process [107]. Injured tissues elicit macrophages and this leads to the production of inflammatory and pro-inflammatory mediators that include, but not limited to, NOS, iNOS and COX-2 [108]. The process begins with the activation of macrophages by tissue injury which increases the amount of NO, iNOS, COX-2, prostaglandin E2 (PGE2) and lipopolysaccharide (LPS) [109,110]. This is most effected by the involvement of macrophages in the regulation of pathways such as the mitogen-activated protein kinase (MAPK) pathway and nuclear factor-kappa B (NF-κB) pathway [110,111]. In event of damage to blood vessels, hemostatic mechanisms must kick in to reduce the flow of blood to the injured site by mechanical vasculature movements and initiation of blood clotting [112].

In events that the initial inflammatory response to tissue damage, called acute inflammation, is unsuccessful, the process matures into chronic inflammation, which may be of detrimental consequences [113]. Progression into chronic inflammation occurs when mechanisms responsible for termination of inflammation are poorly regulated and inflammatory mediators clearance fails [114]. As a result, inflammatory mediators continuously increase and lead to the production of substances that cause

damage to DNA such as ROS and RNS [114]. Tumorigenesis, the development of cancer cells, is among the resulting conditions of chronic inflammation [115].

2.4 Anti-inflammatory agents

The widely reported side effects of NSAIDs serve as a trigger to research directed at discovering novel anti-inflammatory drugs. Apart from the effectiveness of phytochemicals such as flavonoids and polyphenols, plant products are cost effective and exhibit minimum to no toxicity [29,116]. Due to various mechanisms of interaction of phytochemicals with macromolecules, several plant-derived compounds interact with body components in the process contributing to the prevention and relief of undesired effects of inflammation [33]. A wide array of phytochemicals, including tannins, flavonoids (such as Quercetin), saponins, alkaloids and terpenes, were found to be present in a water extract of *Psidium guajava* [117]. *Psidium guajava* extract exhibited exceptional egg albumin denaturation activity, greater than the reference drug, Dichlofenac Sodium [117]. In another study by Zhang *et al* [118], aqueous and methanol extracts of *Pergularia daemia* showed a wide variety of phytochemicals with flavonoids and phenolics being in largest amounts. The same extracts from *P. daemia* exhibited decent anti-denaturation activity against egg albumin and bovine serum albumin [118].

The existence of synergistic anti-inflammatory activity between phytochemicals from various medicinal plants is a stimulating factor in the research focusing on combined extracts [118]. Synergistic interactions of phytochemicals could be achieved through simultaneous action on a single target or interaction with different targets by phytochemicals giving rise to pharmacodynamics and pharmacokinetic interactions respectively [94]. The approach of blending extracts presents an advantage of increased

activity even at low concentrations of extracts, hence avoiding any possible toxicity that could arise if higher concentrations of extracts were used [32,118]. Previous studies directed at evaluating the anti-inflammatory potential of plant derived extracts have greatly recommended blending the extracts valuable approach to take advantage of the possible synergy between phytochemicals [119]. Various concoctions are sold in different countries to assist in alleviating ailments, including pain. A study was done to evaluate the anti-inflammatory potential of herbal concoctions obtained from traders in Ga Maya, South Africa [120]. The concoctions exhibited promising inhibition against COX 1 and COX 2 enzymes.

A wide variety of processes are targeted by different substances that treat inflammation. While investigating the *in vitro* anti-inflammatory potential of sesquiterpenes isolated from *Salvia plebeian*, a TCM plant used to manage inflammation, inhibition of the release of nitric oxide (NO) and tumor necrosis factor α (TNF-α) was observed [121]. The same extracts were reported to display anti-inflammatory activity by regulation of protein expression of inducible nitric oxide synthase (iNOS) and COX-2 [121]. In another study, a TCM product called Qingfei Xiaoyan Wan (QF) repressed the release of both cytokines and chemokines including TNF-α, interleukin 6 (IL-6), interleukin 8 (IL-8) and regulated upon activation, normal T-cell expressed and secreted (RANTES) [122].

The TCM decoction, Xuefu Zhuyu decoction (XFZY), possesses anti-inflammatory activity and is traditionally used to manage traumatic brain injury (TBI) which is usually associated with neuroinflammation, together with angina pectoris and ischemic heart disease [123]. Previous results on the *in vivo* effect of XFZY on mice

neuroinflammation revealed a decrease in tumor necrosis factor TNF-α and IL-1β as well as a reduction in arachidonic acid levels, a precursor molecule of prostaglandins (PGs) and leukotrienes (LTs) production [123]. Both PGs and LTs lead to the initiation of inflammation [123]. In a separate study, the contribution to the anti-inflammatory activity of different constituents identified by a combination of activity index (AI), liquid chromatography – mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR) in a TCM formula called Gui-Zhi-Jia-Shao-Yao-Tang (GZJSYT) was investigated [124]. This approach led to the first time identification of three constituents of three constituents from TCMs with anti-inflammatory activity; glycyrrhisoflavone, glisoflavanone and isoangustone among other already identified compounds [124].

With new discoveries and advanced research, new more effective, less energy and cost consuming assays are under development. Studies directed at comparing anti-inflammatory efficacies of a wide range of substances can now employ the latest assay of using high efficacy exome preparations [125].

2.5 Anti-inflammatory assays

The availability of a wide array of model assays for inflammation ensures versatility and flexibility of researchers in selecting what assay to employ in evaluating the anti-inflammatory potential of various substances. After simulating a biological process by *in vivo* processes and introduction of the test substance, a process or conditions similar to inflammation can be employed to evaluate the ability of the test substance to prevent the progression of inflammation [126]. Alongside a test compound is always a standard compound known for anti-inflammatory activity, with diclofenac sodium the most used standard anti-inflammatory drug as a positive control [127]. Substances with anti-

inflammatory activity display effects ranging from direct protection of proteins from denaturation to inhibition of enzymes that initiate or promote inflammation [128,129].

Inhibition of protein denaturation, membrane stabilization [130,131] and enzyme inhibition [70] by therapeutic substances serve as promising evidence for anti-inflammatory potential of such substances. The release of various inflammatory mediators such as IL-6 and IL-8 from cells may as well be employed to evaluate the ability of substances to counteract initiation of inflammation [11]. *In vivo* membrane stabilization assays which most dominantly employs human red blood cell (HRBC) membrane stabilization simulate the expected stabilization by effective anti-inflammatory agents [125]. The release of enzymes from lysosomes due to membrane breakage that happens as part of the inflammation course leads to the initiation of pathways that contribute to the escalation of the inflammation progression and subsequent deteriorative diseases [129]. It is the similarity between the membranes of the lysosomes and HRBCs that provides the confidence that, substances capable of inhibiting HRBC membrane from lysis may inhibit lysosomal membrane lysis too, acting as anti-inflammatory agents [129].

Protein denaturation associated with inflammation serves as the principle behind the albumin denaturation assay [132]. Protein denaturation that takes place during inflammation stimulates the production of auto-antigens that eventually contribute to for example autoimmune diseases and cancer [129]. Egg albumin [21] or bovine serum albumin (BSA) [129] may be used as *in vitro* protein models that may be denatured by heat. Heating to temperatures as high as 70°C denatures protein thereby simulating protein denaturation that leads to inflammation [127]. Proteins can therefore be treated

with various substances and subjected to heat, after which the extent of denaturation can be used as a measure of the potential of the substance under investigation as an anti-inflammatory agent [127]. This is the basis of the albumin denaturation assay.

Enzyme-based assays can be used to establish the ability of various agents to inhibit the action of enzymes involved in the inflammatory pathway [20]. Phospholipase A2 (PLA2) is an enzyme that cleaves phospholipids into constituent fatty acids including arachidonic acid (AA), the fatty acid essential for the production of prostaglandins and leukotrienes [20,133]. Prostaglandins, produced by prostaglandin synthase, alongside leukotrienes and platelet-activating factor act as inflammatory mediators [133]. Inhibition of PLA2 and COX enzymes that produce AA and prostaglandins by potential therapeutic substances is therefore measured as the extend of the substances to inhibit inflammation [20]. PLA2 assay relies on the ability of PLA2 enzyme to cause rupture red blood cells and the amount of hemoglobin released is measured spectrophotometrically to represent the extent of PLA2 activity [20]. In the prostaglandin synthase (COX) assay, spectrophotometric measurements of gabalin produced from AA provides information on the extent of COX enzyme inhibition which translates to anti-inflammatory activity of the substance under investigation [20].

2.6 The link between inflammation and cancer

The most established relationship between cancer and inflammation is the ability of chronic inflammation to cause cancer among other diseases such as meningitis, atherosclerosis, bronchitis and asthma [99,134]. Contribution of inflammation to cancer is well documented as evidenced by the observation that inflammatory mediators are frequently spotted in tumors and sites of chronic inflammation develop into cancerous

locations [135]. In addition to other cancers, brain tumorigenesis has been outlined in literature as one of the consequences of chronic inflammation [136]. One of the detrimental consequences of chronic inflammation of brain cells is epilepsy [137]. Chronic inflammation in bones leads to the deterioration of bones through the inflammatory disease called rheumatoid arthritis [138].

In general, inflammation-derived cancer is achieved through the action of inflammatory mediators involving tumor-promoting mutations, enhancement of blood vessels development (angiogenesis) [113], apoptosis inhibition and adaptation to the new conditions [139]. In some events, inflammation may be the etiology of subsequent cancer as contrasted by several cases whereby a genetic alteration that promotes cancer initiation causes the inflammation [135]. In either case, the inflammation acts as a strong stimulating process to the initiation and progression of cancer [135], hence any successful efforts to terminate the inflammation may lead to early control or prevention of cancer onset.

The link between inflammation and related cancers is best summarized by two pathways: an intrinsic pathway that simultaneously promotes the inflammation and cancer initiation as well as an extrinsic pathway that promotes inflammation that leads to cancer (Fig. 2) [135,140]. DNA damage and tissue injury respectively are the two initiating processes of the intrinsic and extrinsic pathways. Eventually the two pathways converge and produce cancers an inflammation-related cancer [135]. Inflammatory cytokines mostly spotted in cancer environment do not impose prolonged and specific immunity, and the result is unhindered contribution of cytokines and chemokines to malignancy [141].

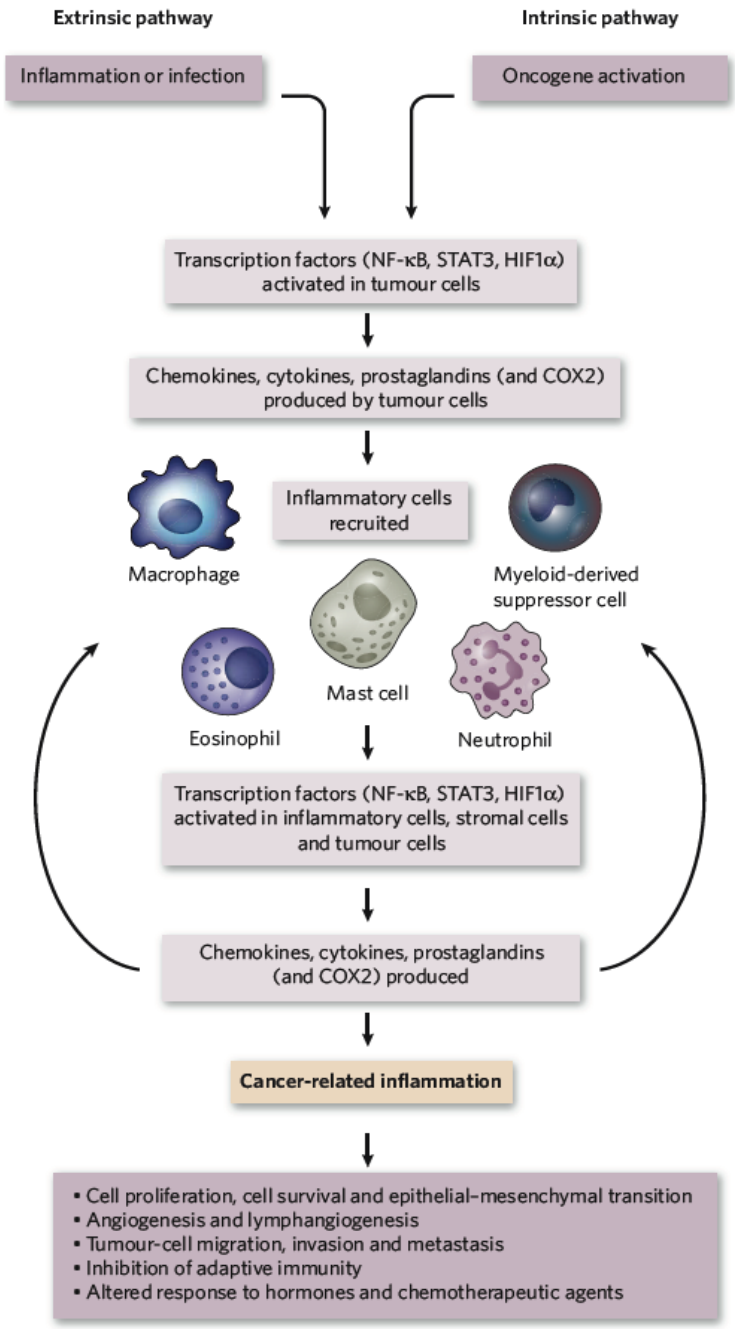


Figure 2. The intrinsic and extrinsic pathways that form a link between cancer and inflammation resulting in cancer-related inflammation. Genetic alterations as well as tissue injury and infections are contributing factors of intrinsic and extrinsic pathways respectively [135].

The immunosuppressive effect of chronic inflammation prevents effective recognition of cancer cell antigens by the immune cells that are present and thus paves the way for cancer initiation and progression [142]. Inflammation may also lead to cancer through non-immune pathways such as generation of ROS, production of angiogenesis-promoting factors as well as metalloprotease production leading to increased cancer spreading [143]. The presence of TNF-α receptors on various tumor and stromal cells and high blood concentrations of TNF-α presents additional explanations to the involvement of inflammation in stimulation of uncontrolled cell division and tumorigenesis [144].

2.7 Cytotoxicity assays

Cytotoxicity assays are developed to evaluate the effect of various substances on the viability of cells as measure of biological safety of such substances [8,145]. Substances that pose toxicity to cells thereby reducing viability are regarded unsafe for use in medical settings as these substances are likely to cause side effects [146]. Employing *in vitro* cytotoxicity assays provides an advantage over *in vivo* assays where high speed and low cost makes the *in vitro* assays more preferred as preliminary assessments for cytotoxicity [147]. Dead cells usually have damaged cell membranes causing cell contents to leak out of the cells while extracellular constituents that would otherwise not cross an intact cell membrane enter the cells [148]. Measurement of the movement of molecules across cell membranes that lost integrity forms the basis of cytotoxicity assays [148]. Introduction of substances that interact with cellular components that leaked out of dead cells or substances that can penetrate the dead cells and produce an observable change can then be used to determine the extent of viability of cells [148].

A wide variety of assays are available to determine the cytotoxic effect, and sometimes the mode of action of the test substance [149]. Various properties or activities that differentiate living cells from dead cells can be used to determine cell viability, including cell membrane integrity, enzyme activity and release specific cellular components from cells, synthesis or uptake of nucleic acids, dye absorption by cells as well as production of adenosine triphosphate (ATP) [150]. On the basis of the type of measurements performed, cytotoxicity assays can be classified as dye exclusion, colorimetric, fluorometric or luminometric assays [147]. Despite the existence of the different categories of assays used in cytotoxicity studies [150], the most commonly used assays are those that employ colorimetric and luminometric analyses carried out in multiple-well plates [148].

Tetrazolium based colorimetric assays employ numerous dyes such as the positively charged MTT as well as negatively charged compounds such as 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl) -2H-tetrazolium (XTT), and WST-1 [148]. The MTT assay relies on the action of enzymes, converting substrates into products that can be quantified [151]. In the presence of viable cells, water soluble tetrazolium compounds such as MTT that is yellow in color undergo redox reactions as a result of actions by mitochondrial enzymes of the dehydrogenase system, ultimately forming dark blue colored, water-insoluble formazan product [148,152]. Coenzymes such as NADH facilitate the transfer of electrons to MTT, thereby reducing it to its formazan product (Fig. 3). The formed formazan collects in the medium as well as inside and around the cells, but needs to be solubilized before the measurement of change in absorbance due to

the MTT reduction [148]. Solubilization of formazan can be achieved through addition of a variety of solvents including acidified isopropanol, dimethylformamide, DMSO or sodium dodecyl sulfate [148].

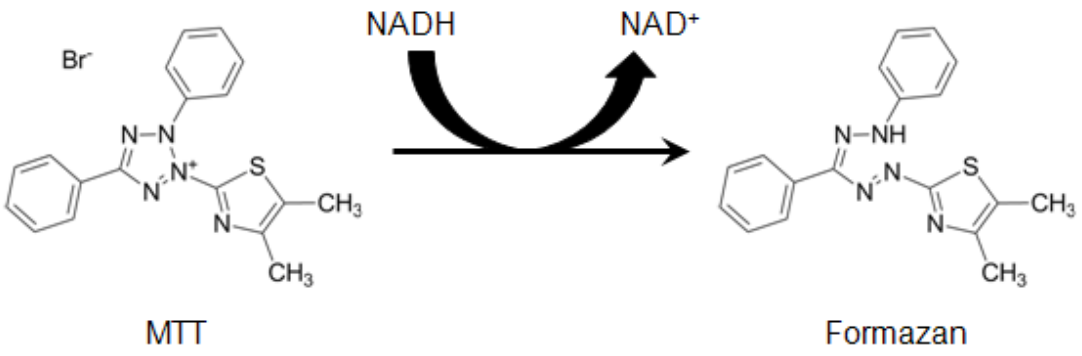


Figure 3. Conversion of MTT dye to formazan by reduction that is assisted by the co-factor NADH. MTT is yellow in color while its formazan product is purple-blue. Adopted from Riss *et al* [148].

Other colorimetric assays include the lactate dehydrogenase (LDH) and SRB assays that measure the LDH enzyme released from dead cells and SRB binding to live, fixed cells respectively [147]. LDH catalyzes the conversion of lactate to pyruvate, (Fig. 2). In the same event, a yellow tetrazolium dye, INT (or 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium), sometimes referred to as iodonitrotetrazolium that is added to the cells is converted to a red tetrazolium dye that can be measured spectrophotometrically [149]. The conversion of INT to the tetrazolium dye is a function of reduction by NADH produced from NAD+ during the conversion of lactate to pyruvate by LDH [150]. The LDH assay is a highly sensitive test that could result in underrepresentation of actual cell viability in the presence of bacteria [153].

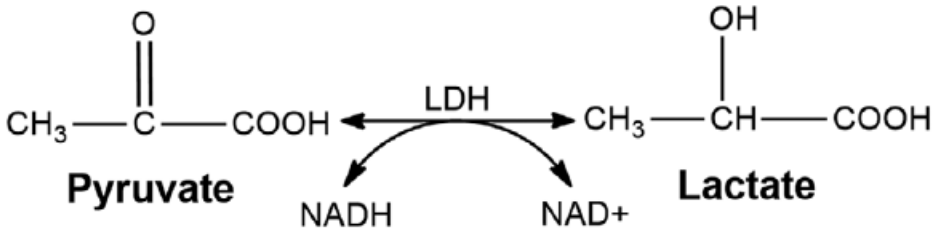


Figure 4. The involvement of LDH enzyme in the formation of pyruvate from lactate alongside the reduction of NAD+ to NADH. Adopted from Senem *et al* [149].

ATP assay is rapid luminometric cell viability determination analysis with high sensitivity [152]. The ATP assay utilizes the enzyme luciferase in the presence of ATP and magnesium ions to oxidize the substrate luciferin to oxyluciferin, in the process producing chemiluminescence that is directly proportional to the number of viable cells [152]. The ATP assay can be used to detect very low cell counts that can be lower than 10 cells/well [147]. Another luminometric assay is the real-time viability assay, that determines the number of viable cells in real time [147]. The ultimate assay that an individual chooses to use depends on the cost effectiveness, speed, reliability and efficiency [147].

3. RESEARCH METHODS

3.1 Research design

Quantitative data on the anticancer of TIB in the form of percent cell viability were obtained from absorbance, indicated by an increase in optical density resulting from conversion of MTT dye into formazan crystals as a result of metabolism by live cells. The *in vitro* anticancer experiments were conducted in a Biosafety Level (BSL) 2 laboratory at the School of Medicine, UNAM. Quantitative anti-inflammatory data were obtained from absorbance changes resulting from heat-induced denaturation of TIB-treated egg albumin.

3.2 Procedure

3.2.1 TIB samples

TIB samples were donated by Prof. Tian Shengxun from the TIB Research Centre in Kenya. The samples were in sealed and well labelled plastic packages (Fig. 5) with codes already assigned to different TIB samples. There were eight TIB samples coded TIB 0, 1, 2, 3, 6, 7, 8 and 9, were obtained and used in this study. Codes of all TIB samples used in this study alongside representative images for each are presented in Table 3. All tablets were first soaked in distilled water for 20 min to soften the sugar coats for ease of removal. After the soaking process, tablets were removed from distilled water and allowed to stand for 20 min to allow water to seep into the tablets. A spatula was used to gently scrape softened sugar coats off the tablets. The tablets with removed sugar coats (Fig 6) were allowed to dry at room temperature and stored at 4°C before further use.

Table 3. The eight TIB samples used in this study and corresponding codes.

Sample	Code	Image
1	TIB 0	
2	TIB 1	
3	TIB 2	
4	TIB 3	
5	TIB 6	
6	TIB 7	
7	TIB 8	
8	TIB 9	

Figure 5. TIB sugar-coated tablets as administered to patients; the color of sugar coats varies among TIB samples (A). Tablets were packaged in well labelled plastic bags (B).

Figure 6. TIB tablets after the removal of sugar coats, leaving only the herbal component that could be responsible for the observed effects of TIB.

TIB extracts were prepared by grinding 3 g (9 - 10 tablets) of tablets without sugar coats to fine powder with a pestle and mortar. Extraction was performed according to the procedure by Manosroi et. al. [154]. The prepared TIB powder was macerated in 60 mL of methanol (MeOH) and dichloromethane (DCM) in a 1:1 ratio for 24 h. The MeOH: DCM solvent system of intermediate polarity was used, dissolving both polar and non-

polar compounds [155]. The mixture was sonicated for 20 min and filtered using Whatman no. 1 filter papers. The obtained extracts were concentrated in a rotary evaporator under reduced pressure and constant temperature of 40 °C. A total of eight extracts were obtained from the eight TIB samples and (w/w) percentage yield was calculated for each extract. The pre-weighed dry extracts were stored at 4°C until further use.

Stock solutions of 20mg/mL of the extracts were prepared in dimethyl sulfoxide (DMSO). The stock extracts were mixed in equal proportions resulting in 5 combination extracts (Comb1-5). The combinations were prepared as administered by Prof. Tian Shengxun in Kenya and Zambia (Table 4). Single TIB extracts as well as combinations of extracts were tested for anti-inflammatory activity as well as for anticancer activity against HeLa and Melanoma cell lines.

Table 4. Combinations of different TIB extracts tested for anticancer and anti-inflammatory activity, making up a total of 5 different combinations.

TIB combination	Composition
1	TIB 0, 1, 2, 3 & 6
2	TIB 1 & 2
3	TIB 3 & 6
4	TIB 6, 7, 8 & 9
5	TIB 6, 7 & 8

3.2.2 Cell lines and cell culture

Cell lines used in the study were donated by the University of the Witwatersrand. Melanoma cells were maintained in MEM while HeLa were maintained in DMEM whereby each of the media was supplemented with 10% fetal bovine serum (FBS). Incubation was done at 37 °C and 5% CO_2 in a Forma Steri-Cycle humidified incubator (Thermo Fisher Scientific). Cell culture flasks (75 cm^2) that have reached 80% cell confluence were trypsinized to detach cells from the bottom of the flask. This was achieved by first removing spent media and washing the flask with 10mL of PBS. Cells were then exposed to 2 mL of trypsin at room temperature before incubation at 37°C for 3 min. The detached cells were removed from the flask with fresh media and cell count was performed on a hemacytometer using trypan blue [156]. After determining cell

concentration, media supplemented with 10% FBS was used to dilute the cells to appropriate cell concentrations.

3.2.3 MTT cytotoxicity assay

The anticancer effect of TIB extracts was determined using the MTT assay as described by Inbathamizth *et al* [157]. This method relies on the conversion of yellow MTT to blue formazan crystals by mitochondrial succinate-tetrazolium reductase which is only active in viable cells [158]. This mechanism allowed the percentage of live cells to be determined by spectrophotometric means. Stock extracts were first diluted in the appropriate complete media, thus reducing the concentration of DMSO that could be toxic to the cells at high concentrations [42]. The extracts were then added to the cell culture plates where serial dilution was performed. HeLa and melanoma cells at concentrations of 1×10^5 cells/mL [50] and 1×10^4 cells/mL [159] respectively were treated with extracts by transferring 100 µL of cells to 100 µL of extracts. Extracts were tested at concentrations of 1.563 – 100 µg/mL. Treatments were incubated in a humidified CO_2 incubator (Thermo Fisher Scientific) at 37 °C, 5% CO_2 for 72 h. After incubation, 100 µL of media in each well was replaced with 80 µL of fresh media and 20 µL of 5 mg/mL MTT and incubated for 2 h. After incubation, 100 µL of acidified propanol was added to each well to dissolve the formed formazan crystals and further incubated for 30 min. Absorbance was read on a SpectraMax M3 plate reader at 550 nm. Absorbance of wells with untreated cells was used as the negative the control while background absorbance was measured with varying concentrations of TIB extracts (1.5625 – 100 µg/mL). Auranofin, Wako Pure Chemical Industries, an antirheumatic

drug with high anticancer activity, tested at varying concentrations (0.195 - 25 μg/mL) was used as a positive control [45].

3.2.4 *In vitro* anti-inflammatory assay

Inhibition of protein denaturation is an activity attributed to anti-inflammatory potential of therapeutic agents. [139,140]. The occurrence of inhibition of protein denaturation, membrane stabilization [141,142] or enzyme inhibition [70] by therapeutic substances is then used as initial evidence for anti-inflammatory potential. The protein denaturation technique was applied in the present study to investigate the anti-inflammatory potential of TIB. Egg albumin was separated from fresh hen's eggs following the salting procedure discussed by Jiang *et al* [160]. Hen's egg contents were transferred to a 1M solution of sodium chloride and stirred vigorously to precipitate the albumin. The resulting mixture was diluted to a ratio of 1:5 which was then used to test for the anti-inflammatory activity. The inhibition of albumin denaturation was evaluated according to the procedure originally reported by Luciano *et al* [25] and modified by Djouonzo *et al.* [161] with slight modifications. Stock extracts (20 mg/mL) were diluted in PBS (pH 6.3) followed by serial dilution. Egg albumin was diluted to a concentration of 16% (w/v) using phosphate-buffered saline (PBS) (pH 6.3) and then, 100 μL of the extracts was mixed with varying concentrations of extracts and the final concentrations of extracts were (3.125 – 200 μg/mL).

Mixtures were incubated at 37 °C in a SpectraMax M2 plate reader for 30 min, and then heated at 70 °C for 15 min in a Salvis lab oven. After cooling the mixture at room temperature for 5 min, absorbance was read at 660 nm on a SpectraMax M2 plate reader. High absorbance represents denaturation of albumin, while low absorbance indicates

that albumin has been protected from denaturation. Wells with untreated albumin were used as the negative control with 0% inhibition (100% denaturation) while background wells contained extracts diluted in PBS at concentrations 3.125 – 200 μg/mL.

3.3 Data analysis

Cell viability was calculated using the formula:

$$\text{Percentage cell viability} = \frac{\text{At - Ab}}{\text{Ac - Ab}} \times 100 \qquad [45]$$

Where: **At** is the absorbance of the treated cells; **Ac** is the absorbance of untreated cells and **Ab** the absorbance of the extract background.

Inhibition of protein denaturation was calculated using the formula:

$$\text{Percent denaturation inhibition} = \frac{Ac - At}{Ac} \times 100 \quad [162]$$

Where: **Ac** is the absorbance of untreated egg albumin and **At** is the absorbance of egg albumin treated with TIB.

Graphpad Prism 8 software was used to compute the 50% inhibitory concentration (IC_{50}) from the percent inhibition of albumin denaturation data. In the anti-inflammatory assay, the IC_{50} represents the concentration at which 50% of egg albumin is denatured in the presence of TIB. The IC_{50} of anticancer data is the concentration at which 50% of cells are viable in the presence of TIB treatment. Since all extracts showed cell viability above 50%, even at the highest concentration tested (100 μg/mL), no IC_{50} calculations were done for the anticancer data. Bott anticancer and anti-inflammatory experiments were carried out in triplicates and were repeated at least 6 times. Percentage viability as well as albumin denaturation data was expressed as mean ± standard error of mean (SEM).

4. RESEARCH ETHICS

Ethical clearance for this study was obtained from the UNAM Research and Ethics Committee. Research permission to conduct the study was granted by the UNAM Centre for Postgraduate Studies. Prof Tian Shengxun granted permission for the use of TIB in this study. Non-hazardous commercial cell lines, commonly used in anticancer studies, were used in the present study. No animal or human subjects have been utilized in this study.

5. RESULTS

5.1 TIB Extraction yield

Extraction of TIB with methanol: dichloromethane (1:1) solvent system yielded a total of eight extracts from eight TIB variations (one extract per TIB variation). Dry mass was used as an indication of efficiency of the extraction. The extraction yielded ranging from 0.2574 – 0.7369 g (Table 5) was obtained.

Table 5. Dry mass yield of extracts obtained from TIB

TIB	Number of tablets used	Mass of tablets (g)	Mass of extract obtained (g)
0	9	3.3514	0.3830
1	10	2.7982	0.3594
2	10	2.9965	0.7369
3	9	2.8600	0.4094
6	9	3.4625	0.2574
7	9	3.9115	0.3134
8	9	3.2447	0.7098
9	9	3.1548	0.5034

5.2 Anticancer activity of TIB against HeLa cells

Percent viability data of HeLa cells after treatment with TIB extracts (TIB 0 – 9) for 72 h is presented in Fig. 7. Cell viability was > 60% for all concentrations tested. The IC_{50} of all extracts was > 100 µg/mL, which was the highest concentration tested for all extracts.

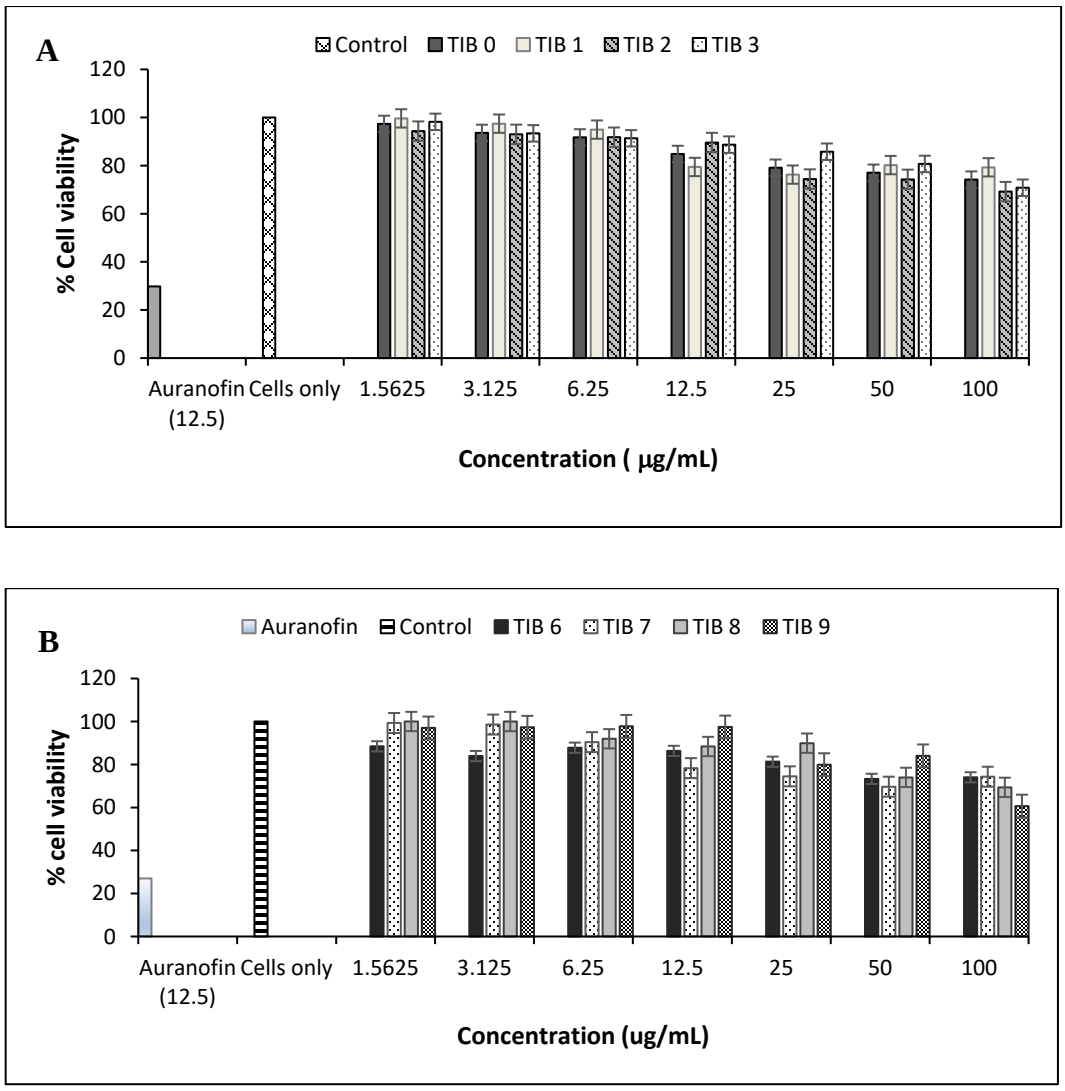


Figure 7. Percent viability of cervical cancer cell line, HeLa, 72 h after treatment with TIB extracts, TIB 0-3 (A) and 6-9 (B), at concentrations of 1.5625-100 µg/mL. Auranofin was used as a positive control (IC_{50}: 2.920 µg/mL). Untreated cells were used as negative control and were considered to be 100 % viable. All TIB IC_{50} values were > 100 µg/mL. Error bars represent mean ± SEM.

5.3 Anticancer activity of TIB against Melanoma cells

TIB extracts were tested against Melanoma cell line to represent its anticancer potential against the skin cancer cell line. Cell viability data of Melanoma cells after 72 h of treatment with TIB 0-9 are presented in Fig. 8. The lowest cell viability of 56.1% was

observed with TIB 3, at a concentration of 100 µg/mL. At extract concentration of 100 µg/mL, TIB 2 treatment resulted in the least reduction of Melanoma cell viability with percent cell viability of 87.7%. All extracts exhibited dose-dependent reduction in cell viability with increased concentration of extracts. No IC_{50} calculations were done for the treatment of Melanoma with the different TIB extracts, as all concentrations resulted in cell viability above 50%, at the highest concentration tested (100 ug/mL).

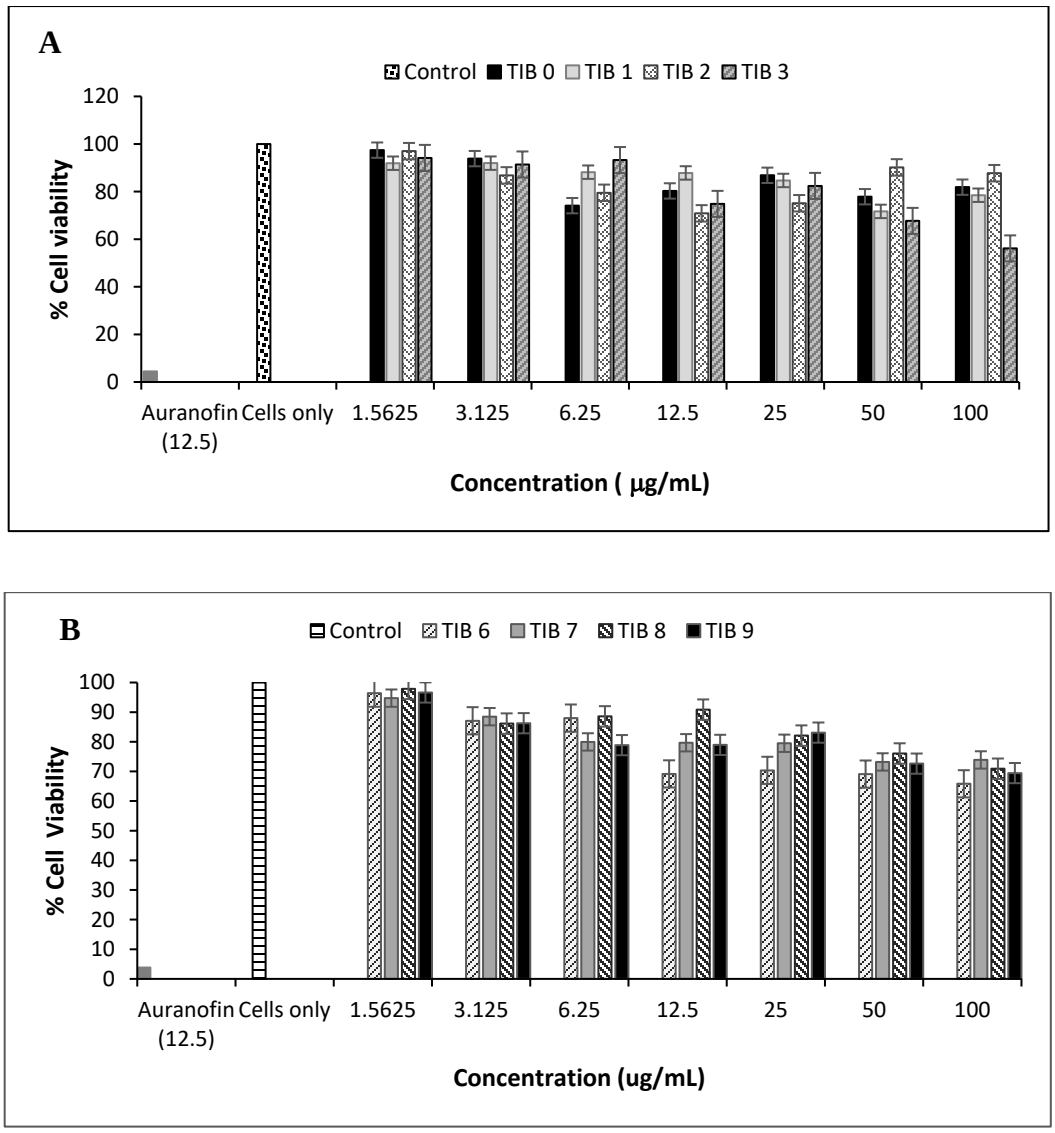


Figure 8. Percent viability of Melanoma cells 72 h after treatment with TIB extracts, TIB 0-3 (A) and 6-9 (B), at concentrations of 1.5625-100 µg/mL. Auranofin (IC_{50}: 1.941 µg/mL) was used as a positive control. Untreated cells were used as negative control and were considered to be 100 % viable. All TIB IC_{50} values were > 100 µg/mL. Error bars represent mean ± SEM.

5.4 Anticancer activity of combined extracts against Melanoma cells

Combined TIB extracts, in equal amounts, were tested against the Melanoma cell line using the MTT assay. Percent viability of Melanoma cells treated with combinations of TIB extracts is shown in Figure 10. Combined TIB extracts showed no cytotoxicity whereby cell viability was close to 100%. At a concentration of 100 µg/mL, TIB Comb3 showed the highest reduction in viable melanoma cells, with viability of 54.4%. TIB comb1 and comb2 showed the lowest melanoma cell viability inhibition, with 80% viability after a 72-h treatment period. The IC_{50} of all five TIB combinations, TIB comb1 – 5 was above 100 µg/mL, the highest concentration used for the MTT assay.

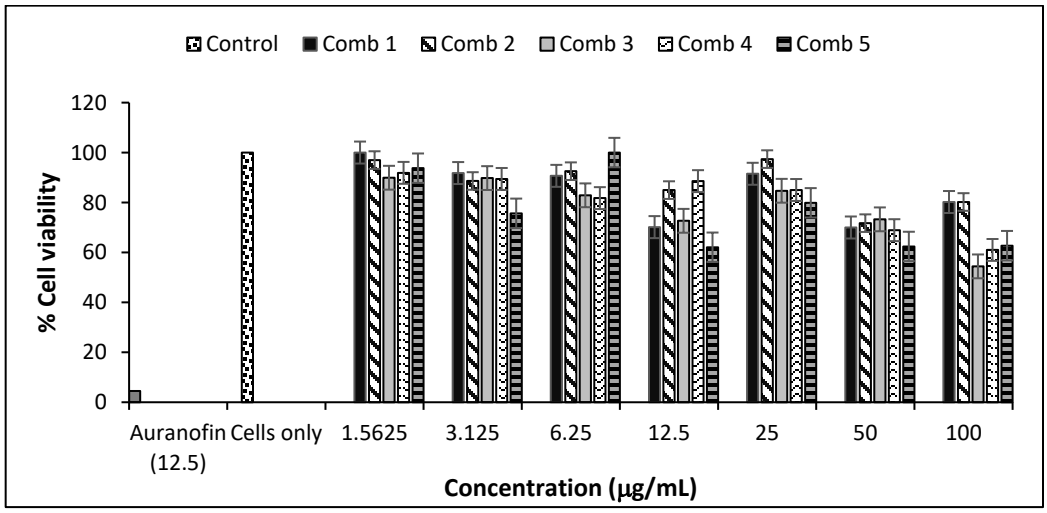
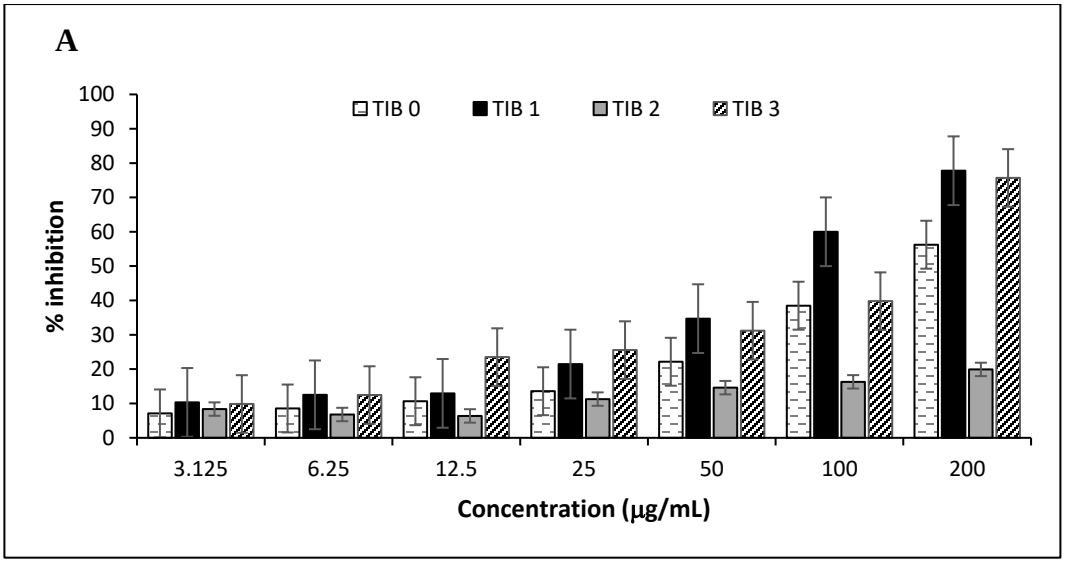


Figure 9. Percent viability of Melanoma cells 72 h after treatment with five combinations of TIB extracts (TIB comb1 – 5) at concentrations of 1.5625-100 µg/mL. Auranofin was used as a positive control (IC_{50}: 1.941 µg/mL). Untreated cells were used as negative control and were considered to be 100 % viable. All TIB IC_{50} values were >100 µg/mL. Error bars represent mean ± SEM.

5.5 Effect of TIB extracts on albumin denaturation

The ability of TIB extracts to inhibit heat-induced denaturation of egg albumin was determined based on the change in absorbance of the egg albumin solution due to protein denaturation after treatment with TIB extracts. The calculated percent inhibition of denaturation of egg albumin by TIB extracts is presented in Fig. 10. All eight extracts exhibited concentration-dependent inhibition of denaturation, whereby the inhibition of protein denaturation increased with increasing extract concentration. TIB 2 showed the least denaturation inhibition, with a percent inhibition of 19.9% at a concentration of 200 μg/mL. Albumin treated with TIB 7 at 200 μg/mL exhibited the highest albumin denaturation inhibition of 91.4%. TIB 6 showed the highest inhibition of albumin denaturation at the concentrations tested compared to other TIB samples, except at 200 μg/mL, where TIB 6 showed the lowest inhibition compared to the other samples.



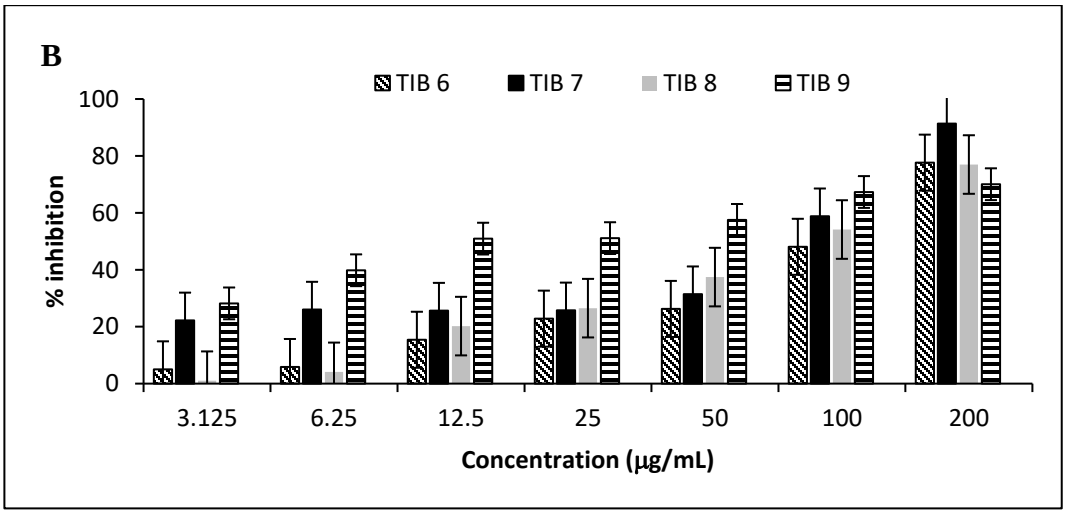


Figure 10. Inhibition of heat-induced egg albumin denaturation by varying concentrations (3.125 – 200 μg/mL) of TIB extracts. Untreated albumin was used as negative control and was considered to be 0 % denatured. All data is presented as mean ± SEM.

All eight extracts showed of IC_{50} values, ranging from 14.53 to > 200 μg/mL (Table 6). TIB 9 displayed the highest albumin denaturation inhibition compared to other TIB

extracts tested, with the lowest IC_{50} value of 14.53 μg/mL. On the other hand, TIB 2 was the least protective against denaturation, displaying an IC_{50} value above 200 μg/mL.

Table 6. IC_{50} data of inhibition of egg albumin denaturation by TIB extracts. IC_{50} data is reported here as mean ± SEM.

TIB	IC_{50} ± SEM (μg/mL)
0	163.84 ± 10.59
1	77.23 ± 4.14
2	> 200
3	136.83 ± 9.35
6	109.77 ± 10.17
7	80.24 ± 3.05
8	76.18 ± 15.33
9	14.53 ± 0.87

5.6 Effect of combined TIB extracts on albumin denaturation

The ability of combined TIB extracts to inhibit the denaturation of egg albumin was carried out to evaluate possible synergistic activity between different extracts. Percent denaturation inhibition data obtained for the five TIB combinations is available in Fig. 11. All five TIB combinations inhibited egg albumin denaturation in a concentration-dependent manner. The increase in inhibition with increasing concentration was highest with TIB comb5. TIB Comb5 showed the highest inhibition (94%) at 200 μg/mL, the highest concentration tested, as compared to all other TIB combinations, Comb1 – 4. Inhibition of egg albumin denaturation at 200 μg/mL was least with treatment with TIB comb3 (88%). Inhibition of albumin denaturation by combined extracts at 200 μg/mL

was above 90% for TIB comb1, 2, 4 and 5 while it was slightly below 90% (89%) for TIB comb3.

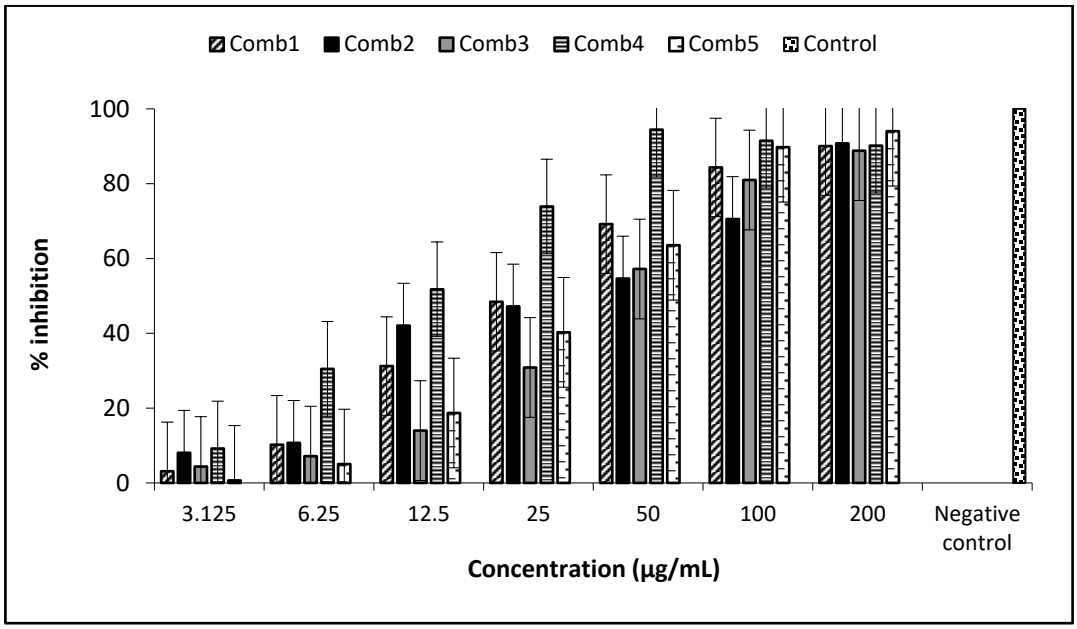


Figure 11. Inhibition of heat-induced egg albumin denaturation by varying concentrations (3.125 – 200 µg/mL) of combinations of TIB extracts. Untreated albumin was used as negative control and was considered to be 100 % denatured. All data is presented as mean ± SEM.

Combinations of TIB extracts showed a slight difference in IC_{50} values that fall between 10 µg/mL and 50 µg/mL. The lowest IC_{50} of 12.30 ± 0.29 µg/mL was recorded for TIB comb4 treatment while the highest IC_{50} value (44.85 ± 2.77 µg/mL) was recorded for TIB Comb3. The IC_{50} values of inhibition of egg albumin denaturation by combined TIB extracts alongside SEM are recorded in Table 7.

Table 7. IC_{50} data of inhibition of heat-induced albumin denaturation by combined TIB extracts reported as mean ± SEM.

TIB comb.	IC_{50} ± SEM (μg/mL)
1	26.92 ± 4.92
2	26.78 ± 3.92
3	44.85 ± 2.77
4	12.297 ± 0.29
5	33.027 ± 0.22

6. DISCUSSION

All eight TIB extracts tested in the present study displayed no cytotoxicity against both HeLa and Melanoma cell lines. A plant extract is considered highly cytotoxic *in vitro* against a given cell line if it displays a CC_{50} value < 1.0 µg/mL, cytotoxic if CC_{50} is between 1.0 and 10 µg/mL, moderately cytotoxic if CC_{50} is between 10 and 100 µg/mL and finally, an extract is non-cytotoxic if it displays CC_{50} > 100 µg/mL [38,163,164]. All TIB samples tested against both HeLa and Melanoma as single extracts and as combination displayed results that indicate that TIB components soluble in dichloromethane/methanol are non-cytotoxic against HeLa and melanoma cell lines [165]. The non-cytotoxicity observed with TIB extracts may be an indication of minimum occurrence of side effects when TIB is administered to patients. It is reported that patients administered with TIB do not report any side effects [2].

Various *in vitro* cytotoxicity studies produced results similar to the findings of the present study, whereby extracts from plants and plant products displayed no cytotoxicity [166,167]. Similarly, Joshanda decoction, a polyherbal formulation used to manage respiratory inflammation was previously found to display mild cytotoxicity [168]. Water extracts from a TCM herb called guduibu previously showed no cytotoxicity against mouse osteoblasts [169]. TIB is traditionally used to manage cancer, but the present *in vitro* evaluation did not shown cytotoxicity towards cancer cells. The reported anticancer activity from the traditional use could therefore be through a different pathway cervical cancer and Melanoma cells directly. It was previously reported that black rice extract was found to inhibit *in vivo* breast cancer proliferation via the caspase activation and membrane depolarization [166]. The black rice extract, however, exhibits *in vivo*

anticancer activity via the inhibition of angiogenesis [166]. This is a representative example of that lead to anticancer activity demonstrated through different pathways by a given agent. Reportedly TIB is believed to treat cancer through the activation of the immune system [2]. Diverse constituents of different Chinese Herbal Medicines (CHMs) used as immune boosters allow the CHMs to exhibit a wide range of biological activities [170].

Since protein denaturation is a contributing factor of inflammation, substances that inhibit protein denaturation are considered suitable candidates for anti-inflammatory evaluation [117,132]. TIB extracts demonstrated high concentration-dependent inhibitory activity against heat-induced denaturation of egg albumin, an indication of the potential of TIB as an anti-inflammatory agent. Of the single extracts, TIB 9 showed the highest activity against albumin denaturation. This could be an indication that TIB 9 contains the highest number of constituents with anti-inflammatory activity. An increase in anti-inflammatory activity of TIB was observed when extracts were tested in combination which may be an indication of synergistic activity between herbal constituents of TIB when extracts are combined. Present findings are in agreement with results from previous studies where medicinal plant extracts displayed synergistic activity when tested in combination [94,118,162]. A previously published study comparing the anti-inflammatory potential of eight herbal remedies and the combined extract formulation of these remedies observed that, the combined extract formulation both in terms of its anti-inflammatory as well as antioxidant activity was more when compared to activities observed when the herbs were tested individually, as a result of synergy between different herbal constituents [171]. Synergistic activity between

constituents of medicinal plants play an important role in reducing dosage required for effective biological activity [118].

Investigating herbal products for more than one biological activity presents an advantage of designing and administrating a single effective drug to patients with multiple morbidities hence the importance of testing TIB for both cytotoxicity and anti-inflammatory potential. Herbal remedies used in different studies have shown promising anti-inflammatory activity alongside minimal cytotoxicity. *Phaleria macrocarpa* fruit extracts were previously assessed for cytotoxicity and anti-inflammatory activity whereby minimal cytotoxicity was displayed against Vero cells and good anti-inflammatory was displayed in the through lipoxygenase enzyme inhibition [165]. Similarly, evaluation of biological activities of methanol extracts from four medicinal plant extracts showed more than one biological activities [172]. Low cytotoxicity and high anti-inflammatory activity were also observed for *Opuntia stricta* extract, indicating a trend of inflammation prevention and minimal cytotoxicity among herbal products [172]. Simultaneous occurrence of minimal cytotoxicity and good anti-inflammatory activities among herbal products observed in previous studies [165,172] corresponds to findings from the present study with TIB.

Since inflammation leads to a wide variety of diseases including cancer, [108,135] anti-inflammatory agents may play a significant role in the prevention of inflammation-associated diseases. Extracts and compounds with anti-inflammatory properties have been noted to display good activity in the management of cancer [173]. Therefore, the anti-inflammatory activity of TIB in the present study is in agreement with the traditional use of TIB against cancer and other ailments [1].

7. CONCLUSION

TIB is traditionally used for the management of different ailments ranging from kidney and heart diseases to cervical cancer and HIV. The cytotoxicity and anti-inflammatory potential of TIB was evaluated and reported in this study. Based on the present findings, it can be concluded that TIB possesses no cytotoxicity towards HeLa and Melanoma cell lines. Results from the anti-inflammatory assay reveal that TIB has the ability to prevent heat-induced albumin denaturation in a concentration dependent manner. Since protein denaturation causes inflammation, the observed ability of TIB to prevent protein denaturation indicates that TIB could be an indication of good anti-inflammatory property. The present study further indicated that combination extracts of TIB may have higher anti-inflammatory potential than single extracts.

8. RECOMMENDATIONS

In the present study, the anticancer activity of TIB was tested against two cancer cell lines (HeLa and Melanoma). Future studies may include non-cancerous cells to evaluate the effects of TIB on non-cancerous cells. Combinations of extracts were not tested on HeLa cells in this study. Future studies may also consider incorporating testing combined TIB extracts on HeLa cells. Aspirin, diclofenac and ibuprofen are anti-inflammatory drugs most commonly used as positive controls in the albumin denaturation assay. Anti-inflammatory assays other than the albumin denaturation assay may be used in future studies. The positive controls for albumin denaturation assay were tested in the present study but did not show the expected results and should be considered in future studies. A negative control, containing untreated egg albumin was included in the present study. Future studies may also consider characterizing extracts from TIB with the aim of understanding its chemical composition. Since TIB is already used in clinical settings, it is recommended that *in vivo* studies may be included in future studies to validate the findings from the present study.

REFERENCES

1. Simengwa C. Tian Immune Booster gins ground as Kenya aids treatment failure rate soars. Times of Zambia. 2017 Jul 28;1–2.

2. Simengwa C. Tian immunity booster : HIVwonder drug. Times of Zambia [Internet]. 2016 Dec 13;1–2. Available from: http://www.times.co.zm/?p=90696

3. Biko R. Germany university endorses herbal HIV immune booster. Standard Digital [Internet]. 2017 Jun 6;1–6. Available from: https://www.standardmedia.co.ke/health/article/2001242411/germany-university-endorses-herbal-hiv-immune-booster

4. Rajendran K, Reddy EV, Khanna A. Anticancer effect of Mesua ferrea extracts on Human Pancreatic Cancer Cell line. Int J Life Sci Sci Res [Internet]. 2016;2(2):198–205. Available from: http://ijlssr.com

5. Omosa LK, Midiwo JO, Masila VM, Gisacho BM, Munayi R, Francisca-Kamakama, et al. Cytotoxicity of 91 Kenyan indigenous medicinal plants towards human CCRF-CEM leukemia cells. J Ethnopharmacol [Internet]. 2016;179:177–96. Available from: http://dx.doi.org/10.1016/j.jep.2015.12.028

6. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. Cell [Internet]. 2011;144(5):646–74. Available from: http://dx.doi.org/10.1016/j.cell.2011.02.013

7. Sharma R, Chandan G, Chahal A, Saini R V. Antioxidant and Anticancer Activity of Methanolic Extract From Stephania Elegans. Int J Pharm Pharm Sci. 2017;9(2):245.

8. Tiralongo E, Uddin SJ, Grice ID. Cytotoxic effects of Bangladeshi medicinal plant extracts. Evidence-based Complement Altern Med. 2011;2011.

9. Newman DJ, Cragg GM. Natural products as sources of new drugs over the last 25 years. J Nat Prod. 2007;70(3):461–77.

10. Patel A, Soni A, Siddiqi NJ, Sharma P. An insight into the anticancer mechanism of Tribulus terrestris extracts on human breast cancer cells. 3 Biotech [Internet]. 2019;9(2):1–10. Available from: http://dx.doi.org/10.1007/s13205-019-1585-z

11. Marquardt P, Seide R, Vissiennon C, Schubert A, Birkemeyer C, Ahyi V, et al. Phytochemical characterization and in vitro anti-inflammatory, antioxidant and antimicrobial activity of Combretum collinum fresen leaves extracts from Benin. Molecules. 2020;25(2):1–18.

12. Nelson SS, Yadav SA, Surendren LK. Evaluation of in vitro anticancer potential in Punica granatum, Psidium guajava, and Vitis vinifera seed extracts. Int J Res Pharm Sci. 2014;10(1):165–9.

13. Jose A, Kannan E, Kumar PRAV, Madhunapantula SRV. Therapeutic potential of phytochemicals isolated from Simarouba glauca for inhibiting cancers: A review. Syst Rev Pharm. 2019;10(1):73–80.

14. Gu S, Pei J. Innovating Chinese herbal medicine: From traditional health practice to scientific drug discovery. Front Pharmacol. 2017;8(JUN):1–5.

15. Artun FT, Karagoz A, Ozcan G, Melikoglu G, Anil S, Sutlupinar N. Anticancer plant extracts on HeLa and Vero cell lines. 2016;21(3):720–5. Available from: https://www.jbuon.com/archive/21-3-720.pdf

16. Bhutia S. Evaluation of In-vitro Anti-Inflammatory activity of Citrus macroptera Montr. Asian J Pharm Clin Res [Internet]. 2020 May 25;13(8):101–3. Available from: https://innovareacademics.in/journals/index.php/ajpcr/article/view/38063

17. Ambriz-Pérez DL, Leyva-Pérez N, Gutierrez-Grijalva EP, Heredia JB. Phenolic compounds: Natural alternative in inflammation treatment. A Review. Cogent Food Agric [Internet]. 2016;2(1). Available from: http://dx.doi.org/10.1080/23311932.2015.1131412

18. Of AA, Uliginosa A, Cass SW, An D, Evaluation I-V, Modak D, et al. Anti-inflammatory activity of Acmella uliginosa (sw.) cass. flower methanolic extract on membrane stabilization and protein denaturation: an in-vitro evaluation. NBU J Anim Sc. 2017;11(January 2018):61–9.

19. Rani AA, Punitha SMJ, Rema M. Anti-Inflammatory Activity of Flower Extract of Cassia Auriculata – an in-Vitro Study. Int Res J Pharm Appl Sci. 2014;4(1):57–60.

20. Eze FI, Uzor PF, Ikechukwu P, Obi BC, Osadebe PO. In vitro and In vivo Models for Anti-inflammation: An Evaluative Review. INNOSC Theranostics Pharmacol Sci. 2019;2(2):3–15.

21. Vinchurkar A, Valsange A, Dama L, Sonawane S, Gaikwad N, Mane P, et al. Evaluation of in-vitro anti-inflammatory activity of crude Lawsonia inermis leaf extract using egg albumin denaturation assay. Trends Biotechnol Res. 2014;8(33):44.

22. Moyosore A, Salisu I, Chemistry I, Shallangwa GA. Comparative study of the

anti-inflammatory properties of Hibiscus Sabdarifa Calyx and Malus Domestica with Ibuprofen. MAYFEB J Chem Chem Eng. 2019;2:1–14.

23. M. Modi C, R. Bhatt P, B. Pandya K, B. Patel H, D. Patel U. Comparative Evaluation of in vitro Anti-Inflammatory Activity of Different Extracts of Selected Medicinal Plants from Saurashtra Region, Gujarat. Int J Curr Microbiol Appl Sci. 2019;8(05):1686–98.

24. Sakat SS, Juvekar AR, Gambhire MN. In-vitro antioxidant and anti-inflammatory activity of methanol extract of Oxalis corniculata linn. Int J Pharm Pharm Sci. 2010;2(1):146–55.

25. Saso L, Valentini G, Casini ML, Grippa E, Gatto MT, Leone MG, et al. Inhibition of Heat-induced Denaturation of Albumin by Nonsteroidal Antiinflammatory Drugs (NSAIDs): Pharmacological Implications. Arch Pharm Res. 2001;24(2):150–8.

26. Williams LAD, O'Connar A, Latore L, Dennis O, Ringer S, Whittaker JA, et al. The in vitro anti-denaturation effects induced by natural products and non-steroidal compounds in heat treated (Immunogenic) bovine serum albumin is proposed as a screening assay for the detection of anti-inflammatory compounds, without the use of animals. West Indian Med J. 2008;57(4):327–31.

27. Kumari CS, Yasmin N, Hussain MR, Babuselvam M. Invitro anti-inflammatory and anti-artheritic property of rhizopora mucronata leaves. Int J Pharma Sci Res. 2015;6(3):482–5.

28. Shallangwa GA, Musa H, Nyaga GT. In-Vitro Evaluation of Ethanolic Extracts of

Zingiber Officinale , Sygzium Aromaticum and their 1 : 1 Extracts Blend on Protein Denaturation During Acute Inflammation. J Progress Res Chem. 2015;1(1):1–8.

29. Shallangwa GA, Dallatu YA, Abechi SE, Shuabu HU. Evaluation of in-vitro anti-inflammatory activity of crude Lawsonia inermis leaf extract using egg albumin denaturation assay. FUW Trends Sci Technol J. 2408;1(2):436–41.

30. Heendeniya SN, Ratnasooriya WD, Pathirana RN. In vitro investigation of anti-inflammatory activity and evaluation of phytochemical profile of Syzygium caryophyllatum. J Pharmacogn Phytochem. 2018;7(1):1759–63.

31. Gunathilake KDPP, Ranaweera KKDS, Rupasinghe HPV. In vitro anti-inflammatory properties of selected green leafy vegetables. Biomedicines. 2018;6(4):1–10.

32. Ayat AA, Shimaa AA, Sahar HE, Mawa IA, Marvit OW, Layla FY, et al. The effect of blending of extracts of Sudanese Adansonia digitata and Tamarindus indica on their antioxidant, anti-inflammatory and antimicrobial activities. J Pharmacogn Phyther. 2019;11(2):28–34.

33. Mahdjar S, Bakka C, Dendougui H, Hadjadj M. Phytochemical profile and In vitro Anti-inflammatory Activity of Anvillea radiata (Coss and Dur) flowers Extracts. Asian J Res Chem. 2020;13(1):44.

34. Elgorashi EE, McGaw LJ. African plants with in vitro anti-inflammatory activities: A review. South African J Bot [Internet]. 2019;126:142–69. Available from: https://doi.org/10.1016/j.sajb.2019.06.034

35. WHO. Cancer 12 [Internet]. Cancer. 2018. Available from: https://www.who.int/news-room/fact-sheets/detail/cancer

36. WHO. Globocan 2012 International Agency for Research on Cancer (IARC) [Internet]. Vol. 876, 2012. 2012. Available from: http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx

37. WHO. Cancer Country Profiles: Namibia. 2014.

38. Kai L, Nur M, Kassim I, Fitrya D, Amir H. Antioxidant activity and cytotoxicity property of extracts from various coastal plants against HepG2 cell lines. Curr Res Biosci Biotechnol. 2020;1(2).

39. Liao YH, Li CI, Lin CC, Lin JG, Chiang JH, Li TC. Traditional Chinese medicine as adjunctive therapy improves the long-term survival of lung cancer patients. J Cancer Res Clin Oncol. 2017;143(12):2425–35.

40. Dahham SS, M Tabana Y. In Vitro Anti-Cancer and Anti-Angiogenic Activity of Essential Oils Extracts from Agarwood (Aquilaria crassna). Med Aromat Plants. 2016;05(04).

41. Zafar SY. Financial Toxicity of Cancer Care: It's Time to Intervene. J Natl Cancer Inst. 2016;108(5):24–7.

42. Houghton P, Fang R, Techatanawat I, Steventon G, Hylands PJ, Lee CC. The sulphorhodamine (SRB) assay and other approaches to testing plant extracts and derived compounds for activities related to reputed anticancer activity. Methods [Internet]. 2007 Aug;42(4):377–87. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1046202307000060

43. Mangis J, Mansur T, Kern K, Schroeder J. Selection of an Optimal Cytotoxicity Assay for Undergraduate Research. Bioscene J Coll Biol Teach. 2019;45(1):24–32.

44. Leelaprakash G, Mohan Dass S. Invitro anti-inflammatory activity of methanol extract of enicostemma axillare. Int J Drug Dev Res. 2011;3(3):189–96.

45. Odeleye T, White WL, Lu J. Cytotoxicity of extracts from New Zealand surf clams against organ cancer cell lines. Biomedicines. 2019;7(2):1–14.

46. Resour PP, Kaur R, Kapoor K. Plants as a source of anticancer agents . J . Nat . 2016;1(January 2011):119–24.

47. Janki Bhulabhai P, Bhulabhai PJ, Janki Bhulabhai P, Patel JB, Piyush P, Parmar RS, et al. Anticancer and Cytotoxic Potential of Aqueous Extract of Triticum aestivum on HeLa Cell Line. J Drug Deliv Ther [Internet]. 2016;6(3):84–9. Available from: http://jddtonline.info

48. Barbieri A, Quagliariello V, Del Vecchio V, Falco M, Luciano A, Amruthraj NJ, et al. Anticancer and anti-inflammatory properties of ganoderma lucidum extract effects on melanoma and triple-negative breast cancer treatment. Nutrients. 2017;9(3).

49. Ramos A, Castro-Carvalho B, Prata-Sena M, Dethoup T, Buttachon S, Kijjoa A, et al. Crude extracts of marine-derived and soil fungi of the genus Neosartorya exhibit selective anticancer activity by inducing cell death in colon, breast and skin cancer cell lines. Pharmacognosy Res. 2016;8(1):8–15.

50. Wu WP, Cao J, Wu JY, Chen H, Wang D. Anticancer activity of Bombyx

batryticatus ethanol extract against the human tumor cell line HeLa. Genet Mol Res. 2015;14(1):79–88.

51. Arjun P, Sivan PSS, Priya SM, Krishnamoorthy M, Balasubramanian K. Phytochemical Analysis and Anticancer Activity of Nelumbo nucifera Floral Receptacle Extracts in MCF-7 Cell Line. J Acad Ind Res [Internet]. 2016;4(July):81–5. Available from: http://jairjp.com/MAY 2016/02 KRUBHA.pdf

52. Arimoto T, Kawana K, Adachi K, Ikeda Y, Nagasaka K, Tsuruga T, et al. Minimization of curative surgery for treatment of early cervical cancer: A review. Jpn J Clin Oncol. 2015;45(7):611–6.

53. Ondua M, Njoya EM, Abdalla MA, McGaw LJ. Anti-inflammatory and antioxidant properties of leaf extracts of eleven South African medicinal plants used traditionally to treat inflammation. J Ethnopharmacol [Internet]. 2019;234(December 2018):27–35. Available from: https://doi.org/10.1016/j.jep.2018.12.030

54. Poonthananiwatkul B, Lim RHM, Howard RL, Pibanpaknitee P, Williamson EM. Traditional medicine use by cancer patients in Thailand. J Ethnopharmacol [Internet]. 2015;168:100–7. Available from: http://dx.doi.org/10.1016/j.jep.2015.03.057

55. Li XXX, Yang G, Li XXX, Zhang Y, Yang J, Chang J, et al. Traditional Chinese Medicine in Cancer Care: A Review of Controlled Clinical Studies Published in Chinese. PLoS One. 2013;8(4).

56. Wang JW, Yang ZQ, Liu C, Chen SJ, Shen Q, Zhang TR, et al. Cancer survivors' perspectives and experience on western medicine and traditional Chinese medicine treatment and rehabilitation: A qualitative study. Patient Prefer Adherence. 2015;9:9–16.

57. Li FS, Weng JK. Demystifying traditional herbal medicine with modern approaches. Nat Plants [Internet]. 2017;3(August):1–7. Available from: http://dx.doi.org/10.1038/nplants.2017.109

58. Harhaji Trajkovic LM, Mijatovic SA, Maksimovic-Ivanic DD, Stojanovic ID, Momcilovic MB, Tufegdzic SJ, et al. Anticancer properties of ganoderma lucidum methanol extracts in vitro and in vivo. Nutr Cancer. 2009;61(5):696–707.

59. Zheng Y, Bai L, Zhou Y, Tong R, Zeng M, Li X, et al. Polysaccharides from Chinese herbal medicine for anti-diabetes recent advances. Int J Biol Macromol [Internet]. 2019;121:1240–53. Available from: https://doi.org/10.1016/j.ijbiomac.2018.10.072

60. Mbele M, Hull R, Dlamini Z. African medicinal plants and their derivatives: Current efforts towards potential anti-cancer drugs. Exp Mol Pathol [Internet]. 2017;103(2):121–34. Available from: http://dx.doi.org/10.1016/j.yexmp.2017.08.002

61. Gulumian M, Yahaya ES, Steenkamp V. African Herbal Remedies with Antioxidant Activity: A Potential Resource Base for Wound Treatment. Evidence-based Complement Altern Med. 2018;2018.

62. van Wyk BE. A broad review of commercially important southern African

medicinal plants. Vol. 119, Journal of Ethnopharmacology. 2008. p. 342–55.

63. Sserunkuma P, McGaw LJ, Nsahlai I V., Van Staden J. Selected southern African medicinal plants with low cytotoxicity and good activity against bovine mastitis pathogens. South African J Bot [Internet]. 2017;111:242–7. Available from: http://dx.doi.org/10.1016/j.sajb.2017.03.032

64. Mothibe ME, Kahler-Venter C, Osuch E. in Vitro Effects of a Commercial Herbal Medicine Used As African Traditional Medicine on Human Neutrophils. African J Tradit Complement Altern Med AJTCAM. 2017;14(3):51–60.

65. Laila U, Akram M, Shariati MA, Hashmi AM, Akhtar N, Tahir IM, et al. Role of medicinal plants in HIV/AIDS therapy. Clin Exp Pharmacol Physiol. 2019;46(12):1063–73.

66. van Vuuren S, Frank L. Review: Southern African medicinal plants used as blood purifiers. J Ethnopharmacol [Internet]. 2020;249(December 2019):112434. Available from: https://doi.org/10.1016/j.jep.2019.112434

67. Agyare C, Boakye YD, Bekoe EO, Hensel A, Dapaah SO, Appiah T. Review: African medicinal plants with wound healing properties. J Ethnopharmacol [Internet]. 2016;177:85–100. Available from: http://dx.doi.org/10.1016/j.jep.2015.11.008

68. Chingwaru C, Bagar T, Maroyi A, Kapewangolo PT, Chingwaru W. Wound healing potential of selected Southern African medicinal plants: A review. J Herb Med [Internet]. 2019;17–18(May 2017):100263. Available from: https://doi.org/10.1016/j.hermed.2019.100263

69. Mehrbod P, Abdalla MA, Njoya EM, Ahmed AS, Fotouhi F, Farahmand B, et al. South African medicinal plant extracts active against influenza A virus. BMC Complement Altern Med. 2018;18(1):1–10.

70. Okur ME, Karadağ AE, Üstündağ Okur N, Özhan Y, Sipahi H, Ayla Ş, et al. In Vivo Wound Healing and In Vitro Anti-Inflammatory Activity Evaluation of Phlomis russeliana Extract Gel Formulations. Molecules. 2020;25(11):1–17.

71. Lawal F, Bapela MJ, Adebayo SA, Nkadimeng SM, Yusuf AA, Malterud KE, et al. Anti-inflammatory potential of South African medicinal plants used for the treatment of sexually transmitted infections. South African J Bot [Internet]. 2019;125:62–71. Available from: https://doi.org/10.1016/j.sajb.2019.06.023

72. Shai LJ, Chauke MA, Magano SR, Mogale AM, Eloff JN. Antibacterial activity of sixteen plant species from Phalaborwa, Limpopo Province, South Africa. J Med plants Res. 2013;7(26):1899–906.

73. Erhabor CR, Erhabor JO, McGaw LJ. The potential of South African medicinal plants against microbial biofilm and quorum sensing of foodborne pathogens: A review. South African J Bot [Internet]. 2019;126:214–31. Available from: https://doi.org/10.1016/j.sajb.2019.07.024

74. Moroole MA, Materechera SA, Mbeng WO, Aremu AO. Medicinal plants used for contraception in South Africa: A review. J Ethnopharmacol [Internet]. 2019;235(February):19–27. Available from: https://doi.org/10.1016/j.jep.2019.02.002

75. Cock IE, Selesho MI, van Vuuren SF. A review of the traditional use of southern

African medicinal plants for the treatment of malaria. J Ethnopharmacol [Internet]. 2019;245(March):112176. Available from: https://doi.org/10.1016/j.jep.2019.112176

76. Twilley D, Rademan S, Lall N. A review on traditionally used South African medicinal plants, their secondary metabolites and their potential development into anticancer agents. J Ethnopharmacol [Internet]. 2020;261(June):113101. Available from: https://doi.org/10.1016/j.jep.2020.113101

77. Cock IE, Van Vuuren SF. A review of the traditional use of southern African medicinal plants for the treatment of fungal skin infections. J Ethnopharmacol [Internet]. 2020;251(December 2019):112539. Available from: https://doi.org/10.1016/j.jep.2019.112539

78. Cock IE, Van Vuuren SF. The traditional use of southern African medicinal plants for the treatment of bacterial respiratory diseases: A review of the ethnobotany and scientific evaluations. J Ethnopharmacol [Internet]. 2020;263(July):113204. Available from: https://doi.org/10.1016/j.jep.2020.113204

79. Liu W, Lu L, Ma C, Yan C, Zhao Z, Mohammadtursun N, et al. The evolution of Traditional Chinese Medicine as a disciplinary concept and its essence throughout history. Tradit Med Mod Med. 2018;01(03):171–80.

80. Yuan H, Ma Q, Ye L, Piao G. The traditional medicine and modern medicine from natural products. Molecules. 2016;21(5).

81. Xu HY, Zhang YQ, Liu ZM, Chen T, Lv CY, Tang SH, et al. ETCM: An

encyclopaedia of traditional Chinese medicine. Nucleic Acids Res. 2019;47(D1):D976–82.

82. Li L, Liu H, Shi W, Liu H, Yang J, Xu D, et al. Insights into the Action Mechanisms of Traditional Chinese Medicine in Osteoarthritis. Evidence-based Complement Altern Med. 2017;2017.

83. Wang Y, Zhang Q, Chen Y, Liang CL, Liu H, Qiu F, et al. Antitumor effects of immunity-enhancing traditional Chinese medicine. Biomed Pharmacother [Internet]. 2020;121(July 2019):109570. Available from: https://doi.org/10.1016/j.biopha.2019.109570

84. Luo H, Vong CT, Chen H, Gao Y, Lyu P, Qiu L, et al. Naturally occurring anti-cancer compounds: Shining from Chinese herbal medicine. Vol. 14, Chinese Medicine (United Kingdom). 2019.

85. Chao J, Dai Y, Verpoorte R, Lam W, Cheng YC, Pao LH, et al. Major achievements of evidence-based traditional Chinese medicine in treating major diseases. Biochem Pharmacol [Internet]. 2017;139:94–104. Available from: http://dx.doi.org/10.1016/j.bcp.2017.06.123

86. Chen B, Zhang M, Huang Z, Zhang H, Xu C, Li J, et al. Chinese Herbal Medicine for Treatment of Hiv/aids-associated Diarrhea: a Protocol of Systematic Review and Meta-analysis of Randomized Clinical Trails. 2020;1–14.

87. Wang X, Fang G, Pang Y. Chinese medicines in the treatment of prostate cancer: From formulas to extracts and compounds. Nutrients. 2018;10(3).

88. Akhalwaya S, van Vuuren S, Patel M. An in vitro investigation of indigenous

South African medicinal plants used to treat oral infections. J Ethnopharmacol [Internet]. 2018;210(May 2017):359–71. Available from: http://dx.doi.org/10.1016/j.jep.2017.09.002

89. Yang B, Xie Y, Guo M, Rosner MH, Yang H, Ronco C. Nephrotoxicity and Chinese herbal medicine. Clin J Am Soc Nephrol. 2018;13(10):1605–11.

90. Zhao X, He X, Zhong X. Anti-inflammatory and in-vitro antibacterial activities of Traditional Chinese Medicine Formula Qingdaisan. BMC Complement Altern Med [Internet]. 2016;16(1):4–11. Available from: http://dx.doi.org/10.1186/s12906-016-1475-4

91. Plitzko I, Mohn T, Sedlacek N, Hamburger M. Composition of Indigo naturalis. Planta Med. 2009;75(8):860–3.

92. Li TM, Yu YH, Tsai FJ, Cheng CF, Wu YC, Ho TJ, et al. Characteristics of Chinese herbal medicine usage and its effect on survival of lung cancer patients in Taiwan. J Ethnopharmacol. 2018;213(91):92–100.

93. Lee DYW, Li QY, Liu J, Efferth T. Traditional Chinese herbal medicine at the forefront battle against COVID-19: Clinical experience and scientific basis. Phytomedicine [Internet]. 2020;153337. Available from: https://doi.org/10.1016/j.phymed.2020.153337

94. Zhou X, Seto SW, Chang D, Kiat H, Razmovski-Naumovski V, Chan K, et al. Synergistic effects of Chinese herbal medicine: A comprehensive review of methodology and current research. Front Pharmacol. 2016;7(JUL):1–16.

95. Liu C, Huang Y. Chinese herbal medicine on cardiovascular diseases and the

mechanisms of action. Front Pharmacol. 2016;7(DEC):1–21.

96. Jiao L, Bi L, Lu Y, Wang Q, Gong Y, Shi J, et al. Cancer chemoprevention and therapy using Chinese herbal medicine. Biol Proced Online. 2018;20(1):1–14.

97. Cary DC, Peterlin BM. Natural Products and HIV/AIDS. AIDS Res Hum Retroviruses. 2018;34(1):31–8.

98. Communication P. Unpublished Documnent. Windhoek; 2019.

99. Tatipamula VB, Vedula GS. In vitro anti-inflammatory and cytotoxicity studies of two mangrove associated lichens, Dirinaria consimilis and Ramalina leiodea extracts. Hygeia J drugs Med. 2018;10(1):16–26.

100. Cao M, Zhang W, Li J, Zhang J, Li L, Liu M, et al. Inhibition of SIRT1 by microRNA-9, the key point in process of LPS-induced severe inflammation. Arch Biochem Biophys [Internet]. 2019;666(December 2018):148–55. Available from: https://doi.org/10.1016/j.abb.2018.12.016

101. Villeneuve DL, Landesmann B, Allavena P, Ashley N, Bal-Price A, Corsini E, et al. Representing the process of inflammation as key events in adverse outcome pathways. Vol. 163, Toxicological Sciences. 2018. p. 346–52.

102. Korniluk A, Koper O, Kemona H, Dymicka-Piekarska V. From inflammation to cancer. Ir J Med Sci. 2017;186(1):57–62.

103. Zarbock A, Kempf T, Wollert KC, Vestweber D. Leukocyte integrin activation and deactivation: Novel mechanisms of balancing inflammation. J Mol Med. 2012;90(4):353–9.

104. Liu YZ, Wang YX, Jiang CL. Inflammation: The common pathway of stress-related diseases. Front Hum Neurosci. 2017;11(June):1–11.

105. Zarbock A, Ley K, McEver RP, Hidalgo A. Leukocyte ligands for endothelial selectins: Specialized glycoconjugates that mediate rolling and signaling under flow. Blood. 2011;118(26):6743–51.

106. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: The leukocyte adhesion cascade updated. Nat Rev Immunol. 2007;7(9):678–89.

107. Malik J, Tauchen J, Landa P, Kutil Z, Marsik P, Kloucek P, et al. In vitro antiinflammatory and antioxidant potential of root extracts from Ranunculaceae species. South African J Bot [Internet]. 2017;109:128–37. Available from: http://dx.doi.org/10.1016/j.sajb.2016.12.008

108. Soonthornsit N, Pitaksutheepong C, Hemstapat W, Utaisincharoen P, Pitaksuteepong T. In Vitro Anti-Inflammatory Activity of Morus alba L. Stem Extract in LPS-Stimulated RAW 264.7 Cells. Evidence-based Complement Altern Med. 2017;2017.

109. Tumer TB, Onder FC, Ipek H, Gungor T, Savranoglu S, Tok TT, et al. Biological evaluation and molecular docking studies of nitro benzamide derivatives with respect to in vitro anti-inflammatory activity. Int Immunopharmacol [Internet]. 2017;43:129–39. Available from: http://dx.doi.org/10.1016/j.intimp.2016.12.009

110. Singh P, Ahn S, Kang JP, Veronika S, Huo Y, Singh H, et al. In vitro anti-inflammatory activity of spherical silver nanoparticles and monodisperse

hexagonal gold nanoparticles by fruit extract of Prunus serrulata: a green synthetic approach. Artif Cells, Nanomedicine Biotechnol [Internet]. 2018;46(8):2022–32. Available from: https://doi.org/10.1080/21691401.2017.1408117

111. Shao-Cong Sun. The non-canonical NF-κB pathway in immunity and inflammation. Nat Rev Immunol. 2017;17(9):545–58.

112. Flier JS, Underhill LH, Dvorak HF. Tumors: Wounds That Do Not Heal. N Engl J Med. 1986;315(26):1650–9.

113. Aguilar-Cazares D, Chavez-Dominguez R, Carlos-Reyes A, Lopez-Camarillo C, Hernadez de la Cruz ON, Lopez-Gonzalez JS. Contribution of Angiogenesis to Inflammation and Cancer. Front Oncol. 2019;9(1399):1–10.

114. Lu H, Ouyang W, Huang C. Inflammation, a key event in cancer development. Mol Cancer Res. 2006;4(4):221–33.

115. Neagu M, Constantin C, Caruntu C, Dumitru C, Surcel M, Zurac S. Inflammation: A key process in skin tumorigenesis (Review). Oncol Lett. 2019;17(5):4068–84.

116. Mahalwal RY y el DVS. in-vitro anti-inflammatory activity of oral poly herbal formulations. Pharma Innov J [Internet]. 2018;7(2):272–6. Available from: https://www.academia.edu/38251123/in-vitro_anti-inflammatory_activity_of_oral_poly_herbal_formulations_rashmi_yadav_iftm_university

117. Kariawasam K, Pathirana R, Ratnasooriya W, Handunnetti S, Abeysekera W.

Phytochemical profile and in vitro anti-inflammatory activity of aqueous leaf extract of Sri Lankan variety of Psidium guajava L. J Pharmacogn Phytochem JPP [Internet]. 2017;6(64):22–6. Available from: http://www.phytojournal.com/archives/2017/vol6issue4/PartA/6-3-159-896.pdf

118. Zhang L, Virgous C, Si H. Synergistic anti-inflammatory effects and mechanisms of combined phytochemicals. J Nutr Biochem [Internet]. 2019;69:19–30. Available from: https://doi.org/10.1016/j.jnutbio.2019.03.009

119. Serrano A, Ros G, Nieto G. Bioactive Compounds and Extracts from Traditional Herbs and Their Potential Anti-Inflammatory Health Effects. Medicines. 2018;5(3):76.

120. Matotoka MM, Masoko P. Phytochemical screening and pharmacological evaluation of herbal concoctions sold at Ga Maja Limpopo Province. South African J Bot [Internet]. 2018;117:1–10. Available from: https://doi.org/10.1016/j.sajb.2018.04.013

121. Zou YH, Zhao L, Xu YK, Bao JM, Liu X, Zhang JS, et al. Anti-inflammatory sesquiterpenoids from the Traditional Chinese Medicine Salvia plebeia: Regulates pro-inflammatory mediators through inhibition of NF-κB and Erk1/2 signaling pathways in LPS-induced Raw264.7 cells. J Ethnopharmacol [Internet]. 2018;210(March 2017):95–106. Available from: http://dx.doi.org/10.1016/j.jep.2017.08.034

122. Hou Y, Nie Y, Cheng B, Tao J, Ma X, Jiang M, et al. Qingfei Xiaoyan Wan, a traditional Chinese medicine formula, ameliorates Pseudomonas aeruginosa-

induced acute lung inflammation by regulation of PI3K/AKT and Ras/MAPK pathways. Acta Pharm Sin B [Internet]. 2016;6(3):212–21. Available from: http://dx.doi.org/10.1016/j.apsb.2016.03.002

123. Xing Z, Xia Z, Peng W, Li J, Zhang C, Fu C, et al. Xuefu Zhuyu decoction, a traditional Chinese medicine, provides neuroprotection in a rat model of traumatic brain injury via an anti-inflammatory pathway. Sci Rep. 2016;6(January):1–14.

124. Wang S, Wang H, Liu Y, Wang Y, Fan X, Cheng Y. Rapid discovery and identification of anti-inflammatory constituents from traditional Chinese medicine formula by activity index, LC-MS, and NMR. Sci Rep [Internet]. 2016;6(July):1–13. Available from: http://dx.doi.org/10.1038/srep31000

125. Sheorain J, Mehra M, Thakur R, Grewal S, Kumari S. In vitro anti-inflammatory and antioxidant potential of thymol loaded bipolymeric (tragacanth gum/chitosan) nanocarrier. Int J Biol Macromol [Internet]. 2019;125:1069–74. Available from: https://doi.org/10.1016/j.ijbiomac.2018.12.095

126. Zhang CR, Aldosari SA, Vidyasagar PSPV, Shukla P, Nair MG. Health-benefits of date fruits produced in Saudi Arabia based on in vitro antioxidant, anti-inflammatory and human tumor cell proliferation inhibitory assays. J Saudi Soc Agric Sci [Internet]. 2017;16(3):287–93. Available from: http://dx.doi.org/10.1016/j.jssas.2015.09.004

127. Sureshkumar K, Maheshwaran V, Dharma Rao T, Themmila K, Ponnuswamy MN, Kadhirvel S, et al. Synthesis, characterization, crystal structure, in-vitro anti-inflammatory and molecular docking studies of 5-mercapto-1-substituted tetrazole

incorporated quinoline derivative. J Mol Struct [Internet]. 2017;1146:314–23. Available from: http://dx.doi.org/10.1016/j.molstruc.2017.05.085

128. Perera HDSM, Samarasekera JKRR, Handunnetti SM, Weerasena OVDSJ. In vitro anti-inflammatory and anti-oxidant activities of Sri Lankan medicinal plants. Ind Crops Prod [Internet]. 2016;94:610–20. Available from: http://dx.doi.org/10.1016/j.indcrop.2016.09.009

129. Verma P, Singh B, Kaur A, Kumar V. In-vitro anti-inflammatory activity and anti-arthritic activity of ethyl acetate extracts of Skimmia anquetilia leaves. J Med Herbs Ethnomedicine. 2020;6:42–4.

130. Gopi KS, Ajith AP, Renish C, Duraiselvan S, Subash MPK. Invitro-Anti Inflammatory and Anti Oxidant Activity of Euphorbia Nivulia. Int J Sci Res Sci Eng Technol. 2018;4(8):443–6.

131. Vijayasanthi M, Doss A, Kumar KA. Evaluation of in-vitro anti-inflammatory and antioxidant activity of Pergularia daemia linn. WORLD J Pharm Pharm Sci [Internet]. 2018;7(7):524–31. Available from: www.wjpps.com

132. Sarvaka S, Galgamuwa GLS, Siriwardene U, Silva ARN, Kumarasinghe N. Testing the Anti-inflammatory Activity of Sri Lankan traditional medicine pill using albumin denaturation method. In: 9th International Research Conference – KDU. 2016. p. 12–5.

133. Mark-Maria Agatemor U, Fred Chiligue Nwodo O, Rita Ozah I. Inhibition of Phospholipase A2 and Prostaglandin Synthase Activities as Possible Mechanisms for the Anti-Inflammatory Effect of Cucumis sativus Fruit Homogenate. Acta Sci

Pharm Sci. 2019;3(7):68–73.

134. Marchio P, Guerra-Ojeda S, Vila JM, Aldasoro M, Victor VM, Mauricio MD. Targeting early atherosclerosis: A focus on oxidative stress and inflammation. Oxid Med Cell Longev. 2019;2019.

135. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Vol. 454, Nature. 2008. p. 436–44.

136. Mostofa AGM, Punganuru SR, Madala HR, Al-Obaide M, Srivenugopal KS. The process and regulatory components of inflammation in brain oncogenesis. Vol. 7, Biomolecules. 2017. p. 1–33.

137. Hiscott PS, Unger WG, Grierson I, Mcleod D. The role of inflammation in the development of epiretinal membranes. Curr Eye Res. 1988;7(9):877–92.

138. Ritschl V, Lackner A, Boström C, Mosor E, Lehner M, Omara M, et al. I do not want to suppress the natural process of inflammation: New insights on factors associated with non-adherence in rheumatoid arthritis. Arthritis Res Ther. 2018;20(1):1–11.

139. Shacter E, Weitzman SA. Chronic inflammation and cancer. Oncology. 2002;1–24.

140. Candido J, Hagemann T. Cancer-related inflammation. J Clin Immunol. 2013;33(SUPPL.1).

141. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet. 2001;357:539–45.

142. Shalapour S, Karin M, Shalapour S, Karin M. Immunity , inflammation , and cancer : an eternal fight between good and evil Find the latest version : Immunity , inflammation , and cancer : an eternal fight between good and evil. J Clin Invest. 2015;125(9):3347–55.

143. Ostrand-Rosenberg S, Sinha P. Myeloid-Derived Suppressor Cells: Linking Inflammation and Cancer. J Immunol. 2009;182(8):4499–506.

144. Balkwill FR, Mantovani A. Cancer-related inflammation: Common themes and therapeutic opportunities [Internet]. Vol. 22, Seminars in Cancer Biology. Elsevier Ltd; 2012. p. 33–40. Available from: http://dx.doi.org/10.1016/j.semcancer.2011.12.005

145. Sharif A, Akhtar MF, Akhtar B, Saleem A, Manan M, Shabbir M, et al. Genotoxic and cytotoxic potential of whole plant extracts of kalanchoe laciniata by Ames and MTT assay. EXCLI J. 2017;16:593–601.

146. Araújo JTC de, Lima LA, Vale EP, Martin-Pastor M, Lima RA, Silva PG de B, et al. Toxicological and genotoxic evaluation of anacardic acid loaded-zein nanoparticles in mice. Toxicol Reports. 2020;7:1207–15.

147. Aslantürk ÖS. In Vitro Cytotoxicity and Cell Viability Assays: Principles, Advantages, and Disadvantages. In: Genotoxicity - A Predictable Risk to Our Actual World [Internet]. InTech; 2018. p. 13. Available from: https://www.intechopen.com/books/advanced-biometric-technologies/liveness-detection-in-biometrics

148. Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, et al.

Cell Viability Assays. Assay Guid Man [Internet]. 2004;(Md):1–25. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23805433

149. Kamiloglu S, Sari G, Ozdal T, Capanoglu E. Guidelines for cell viability assays. Food Front. 2020;1(3):332–49.

150. Adan A, Kiraz Y, Baran Y. Cell Proliferation and Cytotoxicity Assays. Curr Biotechnol Pharm. 2016;17(14):1873–4316.

151. Grela E, Kozłowska J, Grabowiecka A. Current methodology of MTT assay in bacteria – A review. Acta Histochem. 2018;120(4):303–11.

152. Karakaş D, Ari F, Ulukaya E. The MTT viability assay yields strikingly false-positive viabilities although the cells are killed by some plant extracts. Turkish J Biol. 2017;41(6):919–25.

153. Van den Bossche S, Vandeplassche E, Ostyn L, Coenye T, Crabbé A. Bacterial Interference With Lactate Dehydrogenase Assay Leads to an Underestimation of Cytotoxicity. Front Cell Infect Microbiol. 2020;10(September):1–11.

154. Manosroi A, Jantrawut P, Sainakham M, Manosroi W, Manosroi J. Anticancer activities of the extract from Longkong (Lansium domesticum) young fruits. Pharm Biol. 2012;50(11):1397–407.

155. Truong DH, Nguyen DH, Ta NTA, Bui AV, Do TH, Nguyen HC. Evaluation of the use of different solvents for phytochemical constituents, antioxidants, and in vitro anti-inflammatory activities of severinia buxifolia. J Food Qual. 2019;2019.

156. Zhang M, Yan L, Wang GJ, Jin R. Resistin effects on pancreatic cancer

progression and chemoresistance are mediated through its receptors CAP1 and TLR4. J Cell Physiol. 2019;234(6):9457–66.

157. Inbathamizh L, Ponnu TM, Mary EJ. In vitro evaluation of antioxidant and anticancer potential of Morinda pubescens synthesized silver nanoparticles. J Pharm Res [Internet]. 2013;6(1):32–8. Available from: http://dx.doi.org/10.1016/j.jopr.2012.11.010

158. Rajesh A, Doss A, Ps T, Vr M. In-vitro anticancer activity of few plant extracts against. Pharma Innov J. 2019;8(3):38–41.

159. Hazarika I, Das A. Anticancer and Antioxidant Property of Bunium bulbocastanum Fruits Various Fractions. Res Rev A J Pharmacogn [Internet]. 2016;3(1):9–13. Available from: www.stmjournals.com

160. Jiang B, Na J, Wang L, Li D, Liu C, Feng Z. Eco-innovation in reusing food by-products: Separation of ovalbumin from salted egg white using aqueous two-phase system of PEG 1000/(NH 4) 2 SO 4. Polymers (Basel). 2019;11(2).

161. Toukam PD, Tagatsing MF, Tchokouaha Yamthe LR, Baishya G, Barua NC, Tchinda AT, et al. Novel saponin and benzofuran isoflavonoid with in vitro anti-inflammatory and free radical scavenging activities from the stem bark of Pterocarpus erinaceus (Poir) [Internet]. Vol. 28, Phytochemistry Letters. Elsevier; 2018. p. 69–75. Available from: https://doi.org/10.1016/j.phytol.2018.09.006

162. Ghumre S V. Assessment of In-vitro Anti-Inflammatory Activity of Cynodon Dactylon and Acyclovir Showing Synergistic Effect by Albumin Denaturation and Membrane Stabilization Assay. Mod Approaches Drug Des. 2017;1(2):1–5.

163. Njeru SN, Muema JM. In vitro cytotoxicity of Aspilia pluriseta Schweinf. extract fractions. BMC Res Notes. 2021;14(1):1–8.

164. Yosie A, Effendy M a. W, Sifzizul TMT, Habsah M. Antibacterial, radical-scavenging activities and cytotoxicity properties of Phaleria macrocarpa (Scheff.) Boerl. leaves in HepG2 cell lines. Int J Pharm Sci Res. 2011;2(7):1700–6.

165. Isa AI, Saleh MIA, Abubakar A, Dzoyem JP, Adebayo SA, Musa I, et al. Evaluation of anti-inflammatory, antibacterial and cytotoxic activities of *Cordia africana* leaf and stem bark extracts. Bayero J Pure Appl Sci. 2016;9(1):228.

166. Hui C, Bin Y, Xiaoping Y, Long Y, Chunye C, Mantian M, et al. Anticancer activities of an anthocyanin-rich extract from black rice against breast cancer cells in vitro and in vivo. Nutr Cancer. 2010;62(8):1128–36.

167. Yan LL, Zhang YJ, Gao WY, Man SL, Wang Y. In vitro and in vivo anticancer activity of steroid saponins of Paris polyphylla var. yunnanensis. Exp Oncol. 2009;31(1):27–32.

168. Khan H, Khan MA, Abdullah. Antibacterial, antioxidant and cytotoxic studies of total saponin, alkaloid and sterols contents of decoction of Joshanda: Identification of components through thin layer chromatography. Toxicol Ind Health. 2015;31(3):202–8.

169. Liu H, Chen R, Jian W, Lin Y. Cytotoxic and antioxidant effects of the water extracts of the traditional Chineseherb Gusuibu(Drynaria fortunei) on rat osteoblasts. 2001;100(6):383–8.

170. Mothibe ME, Kahler-Venter CP, Osuch E. Evaluation of the in vitro effects of

commercial herbal preparations significant in African traditional medicine on platelets. BMC Complement Altern Med. 2019;19(1):1–12.

171. Joshi P, Yadaw GS, Joshi S, Semwal RB, Semwal DK. Antioxidant and anti-inflammatory activities of selected medicinal herbs and their polyherbal formulation. South African J Bot [Internet]. 2020;130:440–7. Available from: https://doi.org/10.1016/j.sajb.2020.01.031

172. Mehwish S, Islam A, Ullah I, Wakeel A, Qasim M, Khan MA, et al. In vitro antileishmanial and antioxidant potential, cytotoxicity evaluation and phytochemical analysis of extracts from selected medicinally important plants. Biocatal Agric Biotechnol [Internet]. 2019;19(April):101117. Available from: https://doi.org/10.1016/j.bcab.2019.101117

173. Naidu KK, Priya SSA, Bharadwaj VT. In-vitro anti-inflammatory and anticancer activities of Octoblepharum albidum Hedw. Am J Med Nat Sci. 2020;1(1):19–24.

APPENDICES

Appendix A: TIB research permission letter

RESEARCH PERMISSION LETTER

PROJECT TITLE: Anticancer potential of Tian Immunity Booster (TIB): investigating against breast, pancreatic and cervical cancers

TO WHOM IT MAY CONCERN

28th March 2019

This serves to confirm that permission was granted to Dr Petrina Kapewangolo to design a study on anticancer potential of Tian Immunity Booster (TIB). The study will be conducted by Moses Hailume (201408366) as a mini-research project for MSc Industrial Biochemistry. The student is registered at the University of Namibia for the programme indicated. TIB samples have been provided for this study.

Yours faithfully

Prof Tian Shengxun
TIB Research Center
Nairobi Kenya
Tel: +254708399380
E-mail: tibaidssolution@gmail.com
tianshengxun@163.com