



IN-VITRO THERAPEUTIC BIOACTIVITY TESTING OF *Medicago sativa L.* (Alfalfa/Lucerne)

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A Treatise

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DECLARATION OF ORIGINAL WORK

I, Thapelo Simon Makhele (Student number: _____) hereby declare that all the work submitted in this project report titled IN VITRO THERAPEUTIC BIOACTIVITY TESTING OF *MEDICAGO sativa L.* (alfalfa/lucerne) is my original and independent academic effort that was carried out under the able supervision of scientists: Dr PM Makhoahle and Professor SS Mashele, and it is hereby submitted to the CUT for the fulfilment of the degree: Master of Health Sciences – Biomedical. I confirm that I know the meaning of plagiarism, and this work has never been presented or submitted to another institution for any other degree or part of a qualification, by me or by any other person.



August 2023

Thapelo Simon Makhele (Delta-1-Actual)

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This treatise is dedicated to my late mother ‘MaThabang Makhele and my father Daniel Makhele. And also, to my family: my late brother Thabang, Theko, Tiisetso (Makhele Makhele), Serame (Icebound), Ntswaki, Mpho, Mamello, Naledi, Lebohang, Lisema, Omolemo, and the more that will be born later.

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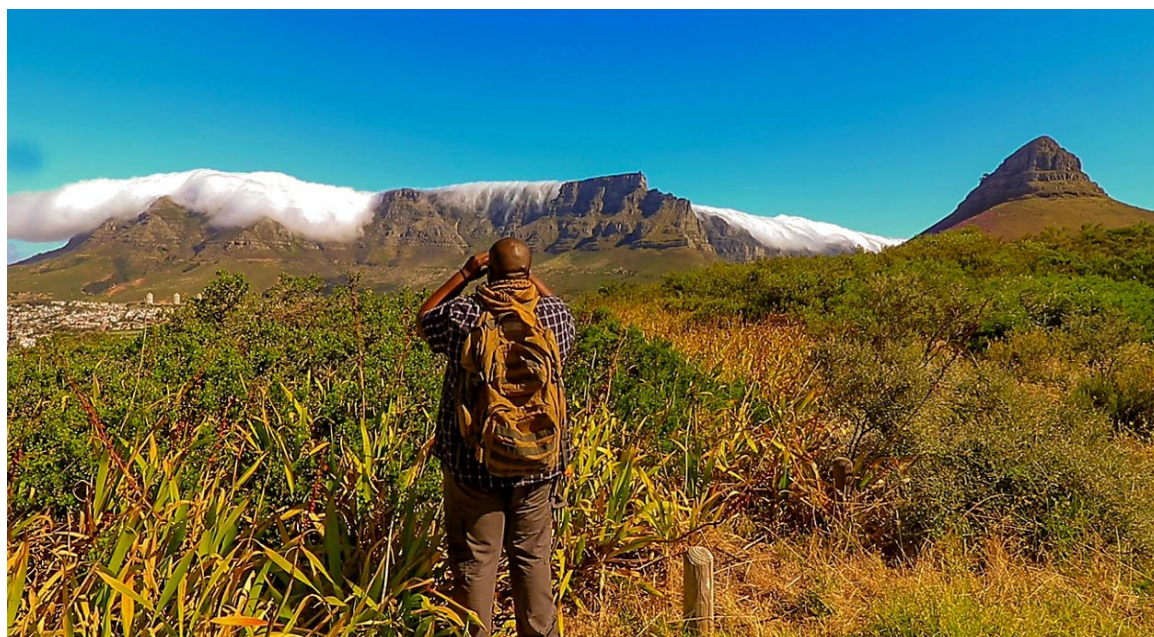
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LIST OF ABBREVIATIONS

AICR	American Institute for Cancer Research
AMR	Anti-Microbial Drug Resistance
COVID-19	Corona Disease of 2019
CSIR	The Council for Scientific and Industrial Research, South Africa
CVD	Cardiovascular Diseases
DPPH	2,2-diphenyl-1-picrylhydrazyl
ECACC	European Collection of Authenticated Cell Cultures, UK
EGCG	Epigallocatechin gallate
IARC	International Agency for Research on Cancer
LPS	Lipopolysaccharide
MABA	Microplate Alamar Blue Assay
Medicago sativa L.	Alfalfa/ Lucerne
MIC	Minimum Inhibitory Concentration
MTT	3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide
NCI	National Cancer Institute, U.S. Department of Health, and Human Services
NSAID	Nonsteroidal anti-inflammatory drug
ROS	Reactive Oxygen Species
RSO	Rick Simpson oil
SAHRPA	South African Health Products Regulatory Authority
SDGs	Sustainable Development Goals
SRB	Sulforhodamine B Assay
THC	<i>Delta-9 tetrahydrocannabinol</i>

PUBLICATIONS FROM THIS TREATISE

1. Makhele, TS. Makhoahle, PM. Mashele, SS. 2022. In-vitro glucose-utilization activity testing of *Medicago sativa* L. (alfalfa) leaf extracts in L6 and HEPG2/C3A cells. *Current topics in Pharmacology*, 26:105-113. Available at http://www.researchtrends.net/tia/article_pdf.asp?in=0&vn=26&tid=11&aid=7034
2. Makhele, TS. Makhoahle, PM. Mashele, SS. 2023. In-vitro bioactivity testing of *Medicago sativa* l. leaf for anti-microbial, ad cytotoxicity screening against Vero cells. *Journal of Advanced Pharmacy Education and Research* 13(2):71-77. Published by SPER publication. <https://doi.org/10.51847/Uj8zZ3w5DT>
3. Makhele, TS. Makhoahle, PM. Mashele, SS. 2023. Laboratory-based anti-inflammatory and DPPH scavenging bioactivity testing of five *Medicago sativa* l. (alfalfa) leaf extracts. *Current topics in Pharmacology*. Vol 27, 2023. http://www.researchtrends.net/tia/article_pdf.asp?in=0&vn=27&tid=11&aid=7200
4. Makhele, TS. Makhoahle, PM. Mashele, SS. 2023. Anti-neoplastic and cytotoxicity potency measuring of five *Medicago sativa* L. leaf extracts towards melanoma (UACC62), Breast (MCF7), Prostate (PC3), and colon (HCT116) cancer cells. *Pharmacognosy Journal*, 15(5). [DOI: 10.5530/pj.2023.15](https://doi.org/10.5530/pj.2023.15).

INTRODUCTION

IN VITRO THERAPEUTIC BIOACTIVITY TESTING OF *Medicago sativa* L. (alfalfa/lucerne)



2015. The Candidate Thapelo Makhele receiving one-on-one lecture @ the University of Stellenbosch by Horticulturist, Botanist, and *Ngaka-chicha* (Medicine Man) who was a PhD candidate at the time. The lecture was practical; it included a walk around Stellenbosch University's Botanical Gardens, collecting species of medicinal importance.

A drug (*moriana*) is a natural or a synthetic material that affects humans' physiology and psychology (behaviour and mood) when consumed. Therapeutic drugs are compounds that are used for the purpose of preventing, curing/healing, treating, and diagnosing of diseases. This treatise is about the laboratory-based testing of therapeutic biological activity (bioactivity) of *Medicago sativa L* leaf extracts. *Medicago sativa L.* is a flowering perennial legume of medicinal, environmental, and agricultural importance that is known around the world by different names. The name Alfalfa is commonly used in America and some parts of North Africa, while in South Africa, New Zealand, Australia, and the United Kingdom the names Lucerne and Holy hay are commonly used to identify the father of all foods: *Medicago sativa*. The leaf is safe for human and animal consumption and medicinal uses includes as an aperient, haemostatic, antiscorbutic, oxytocic, tonic, nutritional supplement, anti-diabetic, and many more. The plant's therapeutic benefits are well known or ethno-pharmaco-botanically established; there exists a lack of empirical evidence in literature to validate the claims. This treatise records the therapeutic bioactivity measuring of *Medicago sativa L.* leaf extracts towards hyperglycaemia (DM), cancer, pathogenic micro-organisms, oxidative stress, and inflammation relief for the first time.

As a response to the existing global burden of diseases, the United Nation's SGD3 aims to ensure healthy lives and to promote well-being for all people at all ages by the year 2030. The urgent need for developing of therapeutics is motivated by the novel emerging infectious diseases, for example, the COVID-19 pandemic, drug resistance to currently existing clinical drugs, the high cost of medicines, the negative side effects induced by current clinical therapeutics, and the evident unequal access to healthcare and medicines. To satisfy the urgent SDG3, new and novel therapeutics are required for combating the persistent rise in premature morbidity and mortality caused by the top ten global causes of disease.

For therapeutic drug development, medicinal plants remain the leading source of ingredients due to their chemical, structural and compound diversity. The ancient medical care system relied on the knowing of natural sources of therapeutics, and still today more than half of the clinical drugs are derived or are based on plants. The other sources of therapeutics are animals, minerals, microbiological, and synthetic sources. Pharmacological screening of natural sources of therapeutics in in-vitro and in-vivo models is the crucial pre-clinical phase of the drug development process. In the year 2007, the general assembly of the UN adopted Resolution 61/225 whereby 14 November of every year is designated as World Diabetes Day as a recognition of the urgent need for novel therapeutics to curb the existing rise in DM

prevalence. Diabetes is regarded to be main cause of blindness, kidney failure, heart attack, stroke, and limb amputations. The modern lifestyle promotes the development of hyperglycaemia, which is the hallmark symptom of DM. Chapter II of this treatise measures the glucose-utilization activity in L6 skeletal muscle and HEPG3A liver cells., whereby the hypothesis is that increased glucose utilization that is induced by alfalfa leaf extracts could mean physiologic alleviation of hyperglycaemia. Natural non-drug-based therapeutics are more desirable because of their affordability, accessibility, and the lack of negative side effects or addiction (dependence).

The therapeutic bioactivity of Lucerne extracts was measure against the clinical standards namely metformin and insulin. *Medicago sativa* leaf extracts induced considerable glucose use both at liver and skeletal muscle levels under controlled laboratory conditions, thus potentiating in-vivo, anti-diabetic therapeutic activity. Oxidative stress and low-grade chronic inflammation are related systematic conditions that can promote a plethora of medical conditions. Chapter III of this treatise measures the DPPH scavenging actions and anti-inflammatory screening of *Medicago sativa L* extracts under controlled laboratory conditions to establish therapeutic potential against related medical ailments. Trolox, EGCG, and Aminoguanidine (AG) served as our positive controls (Figures 3.1-3.3). The modern lifestyle is bloated with risk factors that promote excessive production of ROS, consequently promoting uncontrolled oxidative stress that often leads to low grade chronic inflammatory response that is well known to encourage neurodegenerative disorders (Alzheimer's and Parkinson's Diseases), cancers, diabetes, and cardiovascular ailments (hypertension, atherosclerosis, and stroke). Sedentary lifestyle, poor diet and malnutrition, environmental pollutants and toxins, excessive narcotic and alcohol use are prevalent risk factors affecting modernized and urbanized people.

Cancer is a devastating medical condition that was responsible for approximately 10 million deaths in 2020 (WHO, 2022). According to the WHO 30-50% of cancers can be prevented using science-based approaches that including the regular consumption of alfalfa herbal tea, regular physical activity, maintaining a diet rich in phyto-chemo-preventive nutrients, and regular cancer screening for early detection. Existing cancer care is expensive, toxic, and is not accessible to all. Medicinal plants persist to supply chemotherapeutics, for example, paclitaxel, cannabidiol, noscapine, etoposide, vinblastine, and curcumin. Chapter IV of this treatise measures the growth inhibitory and cytotoxicity of alfalfa leaf extracts against four clinical cancer cells (Figures 4.1-4.12). This treatise records the in-vitro bioactivity that

establishes potential anti-microbial, oxidative stress, low-grade chronic inflammation and anti-neoplastic therapeutic relief induced by alfalfa leaf extracts.

Antimicrobial drug resistance in pathogenic organisms poses a significant threat to public health and quashes our clinical ability to treat common infections. Clinical interventions such as surgery are threatened by nosocomial spreading of drug resistant infections. The clinical pipeline of new antimicrobial drugs is dry; furthermore, there is also an unequal access to antibiotics globally. Chapter V of this treatise records the antimicrobial activity and the MIC measuring of alfalfa leaf extracts towards clinical pathogens (Figures 5.3-5.7).

The cytotoxicity screening of alfalfa extracts in green monkey kidney cells (Vero cells) is recorded in Chapter V (Figures 5.8-5.11). Four manuscripts were submitted and following scientific peer review they were accepted and published. Published manuscripts are attached at the end of the thesis as appendices, including abstract acceptances by national and international conferences.

In conclusion, the emerging burden of diseases and resistance to available therapeutics indicates an urgent demand of scientific innovation of healing medicines. The clinical care system is triangular, where clinical symptoms lead to diagnostic testing and then laboratory results will inform the therapeutics to be used. While the traditional healthcare system of times past was circular by nature, healing of ailments or clinical manifestation was more holistic. Prevention of diseases and risk factors is the routine of holistic healing methods and medicinal plants play a central role therein. Our experiments validate scientifically the holistic medicine claims of the therapeutic potency of *Medicago sativa*. The laboratory testing of medicinal plants is the critical step in drug discovery and development research and this treatise contributes new knowledge that can be used for further testing of active compounds from the alfalfa leaf.

The Aim of the Study:

This study was aimed at measuring the therapeutic bioactivity of five Alfalfa leaf extracts under controlled laboratory conditions.

The Objectives of the Study:

1. To determine the Glucose-Utilization activity of five *Medicago sativa L* (alfalfa) leaf extracts.
2. To measure the DPPH scavenging action and anti-inflammatory screening of five Alfalfa leaf extracts.
3. To evaluate the in-vitro neoplastic cell growth inhibition and cytotoxicity bioactivity of Alfalfa.
4. To establish the anti-microbial bioactivity of five leaf extracts against a panel of five pathogenic microorganisms and the cytotoxicity screening against African Green Monkey Kidney cells (Vero Cells).

Ethical Considerations:

This study did not require ethical approval. No humans (or patient data) or experimental animals were used in the study.

The Rationale of the Study:

The ethno-pharmacology of South Africa and the world is well known, but the gap is the scientific evaluation data that validate their therapeutic effects, safety, and dosage. This study fills this gap by evaluating and publishing therapeutic activity testing of *Medicago sativa L*. leaf extracts. Many herbal preparations are on sale in pharmacies and health shops, but most of the products lack empirical data to back the indicated uses.

CHAPTER I: GENERAL REVIEW OF LITERATURE

**In vitro therapeutic bioactivity testing of *Medicago sativa* L.
(alfalfa/lucerne)**

1.1 Introduction and the problem statement

Oxidative stress, low-grade chronic inflammation, neoplasms, pathogenic micro-organism infections, hyperglycaemia, and diabetes mellitus are vital ingredients of the top ten global causes of death, which is a staple of both developed and developing nations of Earth (Sharifi-Rad et al., 2020; WHO, 2020a). The existing epidemiological data show an increasing burden of diseases on global public health and wellness (Arnold et al., 2022). Novel therapeutics (both natural and synthetic) are continuously needed to curb and to prevent the rising emergent diseases. The currently existing clinical drugs are still effective, but they are challenged by their high cost, drug resistance, the negative side effects they cause and the unequal access to medicines. This general review of literature explores the use of plants as sources of healing compounds towards treating diabetes (hyperglycaemia), infections and cancer (Salehi et al., 2019).

Medicinal plants and other natural resources continue to play an essential function in the preservation of human and animal life on the planet Earth (Krakowska et al., 2017). Animals and humans still rely on plants for engineering, food, furniture, shelter, *moriana* (medicine), weapons, and fire (Shintaro et al., 2017). The use of plants and plant-derived compounds for medicinal purposes by humans and animals are old, diverse, and advanced. The discovery, development, and approval of therapeutics is a lengthy multi-phase process that can take up to 15 years (FDA, 2018).

The cost of drug discovery and development has been figured to be around 2 billion USD (\$) for each drug to be approved for clinical use (Sun et al., 2022). The observed failure of the much-needed development of therapeutics means premature death, and disability due to poor public health will increase and continue to exert negative pressure on the economy if no solution is found. The leading causes of drug development of new therapeutics are the lack of clinical efficacy (40 to 50% of failures) and 30% of drug development failures are due to unmanageable toxicity (Sun et al., 2022). The advantage of herbal preparations for use as medicines is that most are not toxic, do not induce severe side effects, and are efficient in dealing with multiple health issues (Salehi et al., 2019).

1.2 The Global Burden of Diseases

The Global Burden of Disease Study 2019 (GBD) is a reliable measure of the state of health and wellness across the world (Arnold et al., 2022). Premature death and disability due to

poor health are a global challenge facing public health currently, despite the evident modern advances in science, technology, engineering, medicine, and mathematics (Arnold et al., 2022). The Institute of Health Metrics and Evaluation's (IHME) Global Burden of Disease study was published in October 2020, where it recorded an upward and a shifting trend of disease burden from 1990 to 2029 (IHME, 2020). The Non-Communicable Diseases (NCDs) accounted for more than 62%, while injuries were reported at 10% and communicable, maternal, neonatal, and nutritional diseases accounting for the 28% (IHME, 2020). The global top ten leading causes of death list consist of seven NCDs (cardiovascular disease and stroke, respiratory disease, malignancy, kidney disease and diabetes) and three communicable diseases: lower respiratory tract infections, neonatal conditions, and diarrhoeal diseases (WHO, 2020a).

Non-communicable diseases (NCDs) are chronic medical conditions that are not caused by an infection that cannot be passed from person to person, and that produces a long-term health and wellness condition that often requires long-term treatment and care (*The Lancet*, 2020). The WHO reported in 2022 that NCDs were responsible for 41 million deaths everywhere, but the larger portion occurring mainly in low- and middle-income countries (WHO, 2020a). NCDs' lethality towards adults of ages 30-69 years was reported to be 15 million people per year; that is to say, 15 million families lost an economically active member prematurely due to poor health and wellness (*The Lancet*, 2019). The risk factors associated with NCDs affect people of all ages and races. Metabolic risk factors, raised blood pressure (hypertension), raised blood sugar levels (hyperglycaemia), being overweight and obese (hyperlipidaemia), and generally poor lifestyle habits (sedentary lifestyle, narcotic, and alcohol abuse) are evidently increasing (*The Lancet*, 2019). The United Nation's Agenda for Sustainable Development Goals (SDG) includes targeting the reduction of the probability of death from NCD's by one third by the year 2030 through innovative prevention, treatment, and health promotion (Siedman, 2017).

1.3 Diabetes Mellitus and Hyperglycaemia

The prevalence of Diabetes Mellitus (DM) continues to increase in South Africa (Zwane et al., 2023). Across all the reviewed articles the consensus is that hyperglycaemia is formidable key risk factor for both communicable and non-communicable diseases such as tuberculosis, neoplasms, DM, and other fatal cardiovascular diseases (Lee et al., 2013; Pearson-Stuttard et al., 2021). The study by Bommer, Sagalova and others estimated the global economic burden

of DM in adults between 2015 and 2030 and predicted that the cost will rise to >2 trillion USD by the year 2030, which is seven years away from the time of writing this treatise (Bommer et al., 2018).

The global incidence of diabetes increased sharply from 108 million to 422 million people with DM in 2014 (WHO, 2020b). A recently published study examined the impact of diabetes on longevity and disability-free life expectancy in South African adults and found that people with DM experienced lower life expectancy and spent greater part of their lives with a disability compared to people without diabetes (Payne et al., 2023). Collin Payne and colleagues claim that in South Africa over 4 million persons live with DM and if no efficient and therapeutically potent natural interventions are established, that figure will rise to 55 million persons or more in Sub-Saharan Africa in the next two decades (Payne et al., 2023). The literature is adamant that efforts aimed at prevention and management of hyperglycaemia and DM are necessary; hence this study's Chapter II explores the glucose utilization activity induced by leaf extracts in L6 muscarine skeletal muscles and HEPG2/C3A cells under controlled laboratory conditions.

The negative impact of DM and hyperglycaemia is observed in the rising prevalence and new diagnosis that is calculated to reach 642 million by 2040 (Mbanya et al., 2010; Guigliano et al., 2018). There are four mechanisms by which hyperglycaemia has been shown to cause diabetic complications, namely activation of the polyol pathway; increased formation of advanced glycosylation end products; activation of Protein Kinase C; and the hexosamine pathways (BrownLee, 2001). The prevalent undesired outcome of diabetic complications includes limb amputations, blindness, or cataracts (retinopathy), heart attacks (vasculopathy), and kidney failure (nephropathy).

The existing literature demonstrates that diabetic complications may arise from reductive and oxidative stress as a result of excessive and persistent production of Reactive Oxygen Species (ROS) (Banach et al., 2015). Adipocytokines in obese individuals are also implicated as the cause of oxidative stress and inflammation, where they can even promote insulin resistance and as thus induce Type-2 diabetes (non-insulin dependent diabetes) and cardiovascular diseases (Ghadge et al., 2018). The central goal in diabetic care is the alleviation of hyperglycaemia to influence the delay or to prevent the onset of DM complications (Fina-Lubaki et al., 2022). For this association between DM, hyperglycaemia, oxidative stress and

low-grade chronic inflammation, this treatise measured the therapeutic potency of alfalfa leaf extracts using reliable, laboratory-based testing models in Chapter II and Chapter III.

The results of our experiments were peer reviewed and published manuscripts are attached hereto as appendices.

Insulin Resistance (IR) refers to a metabolic state where the body's ability to utilize glucose is impaired due to a decreased or abnormal response to insulin and also decreased insulin production (Wei et al., 2008). The risk factors for IR include obesity, physical inactivity (sedentary lifestyle), a diet that is high in carbohydrates, family history, and ethnicity (Rudd et al., 2020). Septic shock is a life-threatening syndrome that follows untreated sepsis, characterized by hypotension and organ dysfunction (Tiwari et al., 2011). Diabetics are known to be more prone to wounds and sores with poor healing, making diabetics more susceptible to nosocomial infections when they are hospitalized (Rudd et al., 2020).

The non-pharmaceutical (non-drug-based alternative medicine) approaches towards diabetic control, treatment, management, and diabetic reversal often depend on the severity of the disease, the age of the subject, their lifestyle, and their physical activity levels (CDC, 2022). The conventional clinical approaches towards diabetic control and management includes the use of sulfonylureas to increase insulin secretion; troglitazone to increase insulin action in fat and muscle cells; metformin to promote insulin mechanisms in the liver; miglitol and acarbose to delay the absorption of carbohydrates from food intake (Stein et al., 2013). There are several concoctions that are used to treat COVID-19 patients with DM, including Dipeptidyl Peptidase-4 (DPP-4) inhibitors, Glucagon-like peptide receptor Agonists, Sodium-Glucose Co-Transporter-2 inhibitors, and other analogues of insulin (Mouhayyar et al., 2020).

The medicinal potential of nature is diverse and has from the beginning of time provided the human and animal health and wellness with sources of beneficial compounds and phytonutrients without causing the negative side effects of synthetic food and drugs. The maximum advantage of plant-based remedies over synthetics is always due to their therapeutic potency versus the side effects they cause, and the wide diversity of beneficial phytochemicals that plants contain (Pagano et al., 2007; Kim et al., 2010; Sirinivasan, 2012). Cock and others conclude that despite the established ethnobotanic knowledge of flora and fauna indicated for DM, the scientific evaluation of anti-diabetic properties of South African medicinal plants is relatively poorly explored (Cock et al., 2020). Therefore, our study is

justified, as it contributes new and urgently needed empirical data that validate anti-diabetic effects of plant extracts for further drug development and testing. However, there are more novel drugs and compounds that are still required to combat DM that are cheap, and that produces low side effects for the user (Coppelli et al., 2020).

1.4 Cancer

Cancer describes an anthology of diseases of higher multicellular organisms, characterized by mutations in the expression of multiple genes, leading to dysregulation of the normal cellular processes of cell division and cell differentiation that has since claimed millions of people globally (Sharifi-Rad et al., 2020). SURVCAN-3 is a multinational collaboration study that measures cancer survivability across Africa, Central and South America and Asia, which has established that disparities in cancer survival across the countries are linked to the country's human development index, availability and efficiency of healthcare and services (Soerjomataram and the SURVCAN-3 collaborators, 2023). The WHO calculated mortality by cancer to be 10 million deaths in the year 2020 (WHO, 2022). The prevalence of cancer is further exacerbated by the modern urbanized lifestyle and environmental pollutants and toxins (WHO, 2022d). The consensus in literature is that about 30% of cancers are preventable and curable if diagnosed early, and that cancer survival is greater in developed countries where access to medicines and healthcare is efficient.

Natural products from natural resources continue to be used in pharmaceutical preparations either as crude extracts, fractions, pure compounds, or analogous compounds (Goodlet et al., 2020). Traditional medical practices of ancient times still possess great therapeutic value and many medicinal plants have been used thus far to synthesize novel therapeutic compounds based on indigenous pharmacopoeias. The most therapeutic bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolic compounds (Scalbert and Williamson, 2000). Chemo-preventive phytochemicals can block or reverse the premalignant stage (initiation and promotion) of the multistep carcinogenesis.

To reduce the rising number of newly diagnosed cancer, new therapeutics that are cheap but effective, and safe but easily accessible are needed urgently. The existing pipeline of new therapeutics approvals by the FDA lists 33 therapeutic compounds that were approved in 2023 and only one of them is indicated towards the treatment of cancer (FDA, 2023). JAYPIRCA is a kinase inhibitor that is indicated for the treatment of adult patients with

relapsed or refractory mantle cell lymphoma that was approved by the FDA on the 27th of January 2023 (FDA, 2023). The clinical safety trial established adverse reactions and laboratory abnormalities, including decreased neutrophil count, decreased haemoglobin, decreased platelet count, fatigue, musculoskeletal pain, decreased lymphocyte count, bruising, and diarrhoea. Medicinal plants are generally preferred for their therapeutic potency, compound diversity, and a lower incidence of adverse reaction experienced by the consumer. The existing lack of newly approved therapeutics does not reflect the true picture of existing anti-cancer compounds derived from nature.

1.5 Anti-Microbial Drug Resistance (AMR) in pathogenic micro-organisms

The pathophysiologic basis concerning the aetiology of diseases has since marvelled traditional healers and scientists' centuries before the times of Lister, Koch, and Pasteur. But most recently, many people have started to discuss not only the effectiveness but also the safety (side-effects, toxicity, etc.) of drug-based disease preventing and disease-treating approaches of our modern medicine. Anti-Microbial Drug Resistance (AMR) describes the condition where the maximal level of the antibiotic drug/agent that can be tolerated by the host does not halt or slow down the growth and development of the infecting organism(s) (Whalen, 2009). The AMR pandemic is a global concern affecting both developed and developing countries. The treatment failures of conventional and available anti-microbial agents are threatened by the persistent multi-drug resistance (Baronikova et al., 2004).

The WHO considers AMR a critical threat to public health that requires a multi-sectoral collaborative work in order to achieve the Sustainable Development Goals (SDGs) by 2030. Anti-biotic susceptibility testing is an effective tool in the discovery of novel bactericidal/bacteriostatic drug agents and therapeutic compounds (Baronikova et al., 2004). The need for effective and cost-friendly therapeutics is urgent and requires a multi-pronged approach that includes looking back into our past for clues and solutions (Gil-Gil, 2019).

The imperial effect of anti-microbial drug resistance is observed in regions such as in South Asia, where every five minutes a new-born child dies from sepsis caused by an antibiotic-resistant pathogen (Bhutta, 2008). The innovations of Alexander Fleming and others are no longer enough a deterrent against infectious agents and the diseases they cause. The pathogens' ability to evolve and adapt has rendered the life-saving magic of many anti-microbial agents as ineffective and hazardous to health and wellness of the sick (Bhutta,

2008). The increasing number of AMR genotypes and phenotypes is an evolutionary response to the widespread use of anti-microbial drugs. and the global connectedness of humans from diverse environments also supports the spreading of pathogens with antimicrobial-drug resistance (Michael et al., 2014). Anti-microbial drug resistance can be inherent or acquired (Whalen, 2009).

Despite apparent advances in healthcare and health technology, nosocomial infections remain a global threat to healthcare across the globe (Ashraf et al., 2020). Common prevalent nosocomial infections include intravenous line-associated bloodstream infections, catheter-associated urinary tract infections, surgical-site infections, and ventilator-associated pneumonia (Khan et al., 2017). Nosocomial pneumonia, vascular access-related bacteraemia and surgical wound infections have caused the most illness and deaths in hospitalized patients, thus making the Intensive Care Units (ICU) the axis of antibiotic resistance (Weinstein, 1998).

The pharmaceutical research and development failed to meet the clinical needs of new and effective anti-microbial agents (Boucher et al., 2013; Kinch et al., 2014). The existing literature poses an urgent need for new and alternative anti-microbial drugs against pathogenic micro-organisms (Luther et al., 2019).

1.6 Conclusion and Recommendations

According to Krakowska et al. (2017), one of the most cultivated perennial fodder crop legumes in the world is *Medicago sativa* L. (also called Lucerne or Alfalfa). *Medicago sativa* L. appears in ancient medicinal herbs/plant books such as the *Compendium of Materia Medica* by Li Shizhen (published in 1596) and it is described in there as the father of all foods: “*alfac-facah*” (Liu et al., 2018). The genus *Medicago* is the part of the *Leguminosae* family that contains 83 known species (Krakowska et al., 2017). *Medicago sativa* L. has evidently been used for many ages in China, India, Africa, and America for medicinal purposes. The plant has been shown to be rich in saponins, flavonoids, coumarins, terpenoids, vitamins and digestive enzymes (Liu et al 2018).



Fig 1.1: Image of *Medicago Sativa L.* (Alfalfa or Lucerne), apparently one the best fodder crops worldwide.

The existing and newly emerging storm of chronic and infectious diseases fuels the demand for novel therapeutics. The healthcare infrastructure of low-income and developing countries is simply not prepared to handle the rising global burden of diseases. The healthcare service in South Africa is unequal and divided into the public and the private sectors. The public sector is funded by the government and caters for more than 70% of the population, while the private sector serves only the few individuals who can afford medical aid and medical insurance. Coupled with the high unemployment rate, access to healthcare is not equal and as thus access to healthcare and medicines of public health importance is disadvantaged.

The United Nations' Sustainable Development Goal number 3 of 17 promises to ensure healthy lives and to promote well-being for all at all ages by the year 2030. To achieve this goal, further scientific exploration of plants of medical importance to establish therapeutic, safety and drug-interaction profiles is recommended. The therapeutic dosage measuring of medicinal plants must be determined in order to achieve repeatable healing results. This treatise establishes new and reliable empirical data (scientifically and linguistically peer reviewed) of the therapeutic bioactivity of the most reliable animal fodder crops, trusted by generations of animal farmers.

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CHAPTER II - EXPERIMENT I:

IN VITRO GLUCOSE-UTILIZATION TESTING OF ALFALFA IN L6 MOUSE SKELETAL MUSCLE AND HEPG2/C3A LIVER CELLS



2022. The Candidate Thapelo Makhele with *Cussonia paniculata* also known as mountain cabbage, a plant of medicinal importance collected on a mountain surrounding Thaba-Nchu (Free State).

2.1 ABSTRACT

Diabetes Mellitus (DM) is a complex, and chronic condition that affects the fate of carbohydrates, fats, and proteins in the human body and is characterized by persistent hyperglycaemia. The global prevalence of diabetes is increasing; the WHO confirmed that diabetes and DM-induced kidney disease caused 2 million deaths in 2019. The current clinical therapeutics used for diabetic care and management include sulfonylureas indicated to enhance insulin secretion, thiazolidinediones to increase insulin action in fat and muscle cells, biguanides (metformin) to lower blood sugar levels, and the alpha-glucosidase inhibitors to delay the absorption of carbohydrates from food intake. The common therapeutic method in Type 1 DM is the use of human-insulin injections to compensate for beta-cell defects. The common drawback of conventional synthetic and chemical therapeutics used in diabetic care is that they never restore the glucose index to normal and they often demand life-long drug dependence and clinical care. The other challenge with existing therapeutics is the reported side effects like the common upset stomach (bloating, gas, and diarrhoea), hypoglycaemia, kidney and liver complications, and weight gain (as well as lowering erectile dysfunction). DM is one of the four major non-communicable diseases (NCDs) that account for increased global mortality and morbidity, and this reason motivates the need for urgent drug development and research in order to accomplish the ideals of the UN's Sustainable Development Goal Number 3. Medicinal plants or plants with therapeutic bioactivity are now a more preferred alternative to synthetic/chemical drugs because of their therapeutic potency, pharmacologically active compound diversity, and because they cause relatively fewer negative side effects. To measure the anti-diabetic potential of Alfalfa, the *in-vitro* glucose-utilization activity induced by five *Medicago sativa L.* leaf extracts was performed in L6 muroid skeletal muscles and C3A/HEPG2 human liver cells. Clinical therapeutics insulin and metformin were used as controls. Because toxicity can impact on the accuracy of our experimental results, the MTT Assay was performed to assess the cytotoxicity induced by our test extracts. The rate of induced glucose-utilization is a measure glucose metabolism that can be interpreted to mean the physiologic alleviation of hyperglycaemia. The leaf extracts of *Medicago sativa L.* induced a considerable glucose-utilization bioactivity towards the L6 and C3A/HEPG2 cells, with the 50µg/ml of the Butanol, water and diethyl-ether extracts exhibiting activity that is comparatively closer to that of metformin. The glucose utilization bioactivity induced by 25-100 µg/ml of our five extracts was consistently half of the activity induced by our control insulin in L6 mouse

skeletal-muscle cells. The evident alfalfa-induced glucose utilization in liver and skeletal cells is a conclusive measure that Alfalfa tea (aqueous extract) can induce physiologic alleviation of hyperglycaemia and as thus be a beneficial non-drug alternative used in the treatment and management/prevention of hyperglycaemia and DM. Further animal-model testing of the anti-diabetic actions of alfalfa to confirm in-vitro and in-vivo correlation of the therapeutic bioactivity testing is recommended.

Keywords: Diabetes Mellitus (DM). Coronavirus Disease 2019 (COVID-19). Metformin. Insulin. Insulin Resistance. Glucose Transporter Type 4 (GLUT-4). Alfalfa (*Medicago sativa* L.) L6 Cells (myoblast skeletal Muscle Cell line). C3A cells (Derivative of human HEPG2 liver cell line). WHO (World Health Organization). Metabolic syndrome. Sepsis. Adipocytokines. Glucose Utilization. Catechins. Phosphate-Buffered Saline (PBS). Bovine Serum Albumin (BSA). Roswell Park Memorial Institute medium (RPMI). Hyperglycaemia. Sedentary Lifestyle.

2.2 BACKGROUND

The World Health Organization (WHO) confirms that diabetes is implicated in causing 1.6 million deaths in the year of 2016 alone. High blood glucose content is attributed to have caused 2.2 million deaths in the year of 2012 (WHO, 2020a). The existing literature demonstrates that diabetic complications may arise from reductive and oxidative stress, through the excessive and persistent production of ROS (Banach et al., 2015). Metabolic syndrome is a complex mix of abnormalities that include obesity, insulin resistance (IR), hypertension, glucose intolerance and dyslipidaemia (Darroudi et al., 2018). Insulin Resistance (IR) refers to a metabolic state where the body's ability to utilize glucose is impaired due to a decreased or abnormal response to insulin and decreased insulin production (Wei et al., 2008). Insulin resistance is a key player in the development of diabetes, cardiovascular disease, and Alzheimer's disease (Saltiel et al., 2001). The treatment, management and prevention of insulin resistance, diabetes, and high percentage of body fat (adipose and visceral) include a radical lifestyle transformation and expensive healthcare interventions such as liposuction or cosmetic abdominoplasty. New and emerging infectious diseases further exacerbate the negative implications of DM and hyperglycaemia (Stasi et al., 2020).

Coronavirus Disease 2019 (COVID-19) is a viral infection that causes a severe acute respiratory syndrome, hyper-inflammatory response, vascular damage, angiogenesis, thrombosis and microangiopathy (Stasi et al., 2020). Diabetes is one of the common problems for patients hospitalized with COVID-19 and it is therein associated with increased risk of morbidity and mortality (Coppelli et al., 2020; Stasi et al., 2020). The common outcome of infections in diabetic individuals is sepsis, which is a systematic bacteraemia and toxemia, often the product of unrecognized and untreated localized and systematic infections (Tiwari et al., 2011). Septic shock is a life-threatening syndrome that follows untreated sepsis, characterized by hypotension and organ dysfunction (Tiwari et al., 2011). Diabetics are more prone to wounds and sores with poor wound healing, this makes diabetics more susceptible to nosocomial infections. The existing therapeutics indicated for DM and hyperglycaemia control are effective but too many negative side effects have been recorded.

The reported negative side effects (gastrointestinal disturbance, hypoglycaemia, weight loss or gain, skin rashes, insomnia, and fatigue) caused by clinical drugs motivates the screening of medicinal plants for therapeutic potential. The cost of medicines and medical care motivates the use of non-drug alternative therapeutics such as Alfalfa herbal tea for the prevention and treatment of hyperglycaemia (Golay and Ybarra, 2005). There are many novel therapeutic methods in testing, such as the use of microcapsules carrying pig-derived islets of Langerhans, which are then implanted beneath the skin of a diabetic patient to restore glucose homeostasis (Kim et al., 2010). Mesenchymal Stem Cell therapy is another promising therapy for DM Type 1, where the transplantation of hematopoietic stem cells has been demonstrated to improve the functioning of beta cells in newly diagnosed Type 1 patients (Kim et al., 2010). Also, there are other experiments where induced pluripotent stem cells (iPS) can be generated from Type 1 diabetics by reprogramming their adult fibroblast with transcription factors like Octamer-binding transcription factor 4, SOX2, and KLF4. These cells have been developed into what it is known as diabetes-induced pluripotent stem cells (DiPS) and they can differentiate into insulin-producing cells (Kim et al., 2010). DM and high blood sugar content are devastating public health concerns of our current times, and innovative efforts are continuing to find a permanent solution for chronic diseases. Insulin is the central player in blood glucose homeostasis, and induced pluripotent stem cell technology offers us the chance to regenerate insulin-producing cells, but herbal preparations are still preferred in DM prevention and glycaemic control.

The advantage of plant-based remedies is due to their therapeutic-potency to negative side-effect ratio that is attributed to the wide diversity of phytochemicals that they contain (Akhani et al., 2005). The active catechins (epicatechin gallate, epigallocatechin gallate) contained in green tea have been demonstrated to inhibit the activity of Glucose Transporter 1 (GLUT-1), which is excessively expressed in cancer cells (Buyukbalci and Sedef, 2008). This observation indicates the mechanism by which tea polyphenols can act as competitive inhibitors of glucose uptake through binding to GLUT-1 (Buyukbalci and Sedef, 2008). This chapter will determine the glucose-utilization bioactivity that will be induced by alfalfa leaf extracts in L6 and C3A/HEPG2 cells.

2.3 MATERIALS AND METHODS

in-vitro Glucose Utilization testing of Alfalfa leaf extracts in L6 and HEPG2/C3A Cells.

2.3.1 About the Plant Material and Extraction

The plant material (dried *M. sativa* L. leaves) were purchased from a nursery and medicinal plant farm North of Pretoria (South Africa) with the Batch Number MH 71(10kg). The plant material was cultivated by Zizameleni Farming based in Mamogaleskraal, Northwest Province of South Africa. The test material was cultivated using regenerative natural farming principles and the fertilizers used were all natural and certified organics. The material was air-dried and stored in a cool dry area away from light and heat. The dry, grassy, and pale green leaves were extracted using five solvents: Butanol, Diethyl-ether, Hexane, Methanol and Water. Sixty (60) grams of powdered plant material was extracted in 1 litre (1000 mL) of every solvent. The extracts were filtered using the Buchner funnel and Whatman no.1 filter paper. The extracts were frozen at -40 °C and freeze-dried for 48 hours at a yield of 9 g of dried extracts. The dried extracts were stored at -4 °C until analysis.

2.3.2 The C3A/HEPG2 and L6 Cellular differentiation and Materials used.

C3A/HEPG2 cells were grown in EMEM (Minimum Essential Eagle's Media) supplemented with 10% FCS and sub-cultured by trypsinisation. Metformin was used as a positive control. The L6 cells are routinely grown in low glucose Dulbecco's Modified Eagle Medium (DMEM). They were then supplemented with 10% Foetal Calf Serum (FCS) and sub-cultured by trypsinisation.

2.3.3 Glucose Utilization in C3A/HEPG2 and L6 cells Assay Procedure

The C3A/HEPG2 cells were seeded into 96-well plates at a density of 5 000 cells/well. At least one row was kept devoid of cells to represent the no-cell control. Cells were incubated for an additional 2 to 3 days to become confluent. Cell cultures were incubated at 37° C in a humidified atmosphere with 5% CO₂. The HEPG2 cells were replenished with growth medium every 2-3 days, consisting of RPMI 1640 medium that was supplemented with 10% fetal calf serum. On day three after seeding, 10 µL test extracts at the concentrations of 25 µg/mL and 100 µg/mL was added to each well.

The spent culture medium from differentiated cells was removed and replaced with fresh medium containing 25 µL of incubation buffer. Metformin (0.1 µg/mL) was used as positive control. The treatment was continued for 48 hours. Spent culture medium was then removed, and the cells washed once with PBS. Fifty micro-litres of 8 mM glucose solution (RPMI medium diluted with PBS and supplemented with BSA to a final concentration of 0.1%) were added. The plates were then returned to the incubator. After 3 hours, 5 µL were transferred into a new plate and 200 µL glucose assay reagent (glucose oxidase/oxidase colorimetric reagent) were added and incubated at 37 °C for 10-20 minutes. The absorbance was measured at 492 nm using the BioTek® SYNERGY Mx fluorometer (Winooski, VT, USA). Glucose utilisation was calculated as the difference between the mean of the no-cell wells and the test sample and expressed as a percentage of the untreated control.

The L6 cells were then seeded into 96-well plates at a density of 5 000 cells/well. At least one row was kept devoid of cells to represent the no-cell control. When approximately 80% confluent, the medium was replaced with differentiation medium (DMEM containing 2% horse serum). The cells were then incubated for an additional 5 days at 37° C in a humidified atmosphere with 5% CO₂ to allow sufficient time for full differentiation. 10 µL test extracts at the concentrations of 25 µg/mL and 100 µg/mL. The treatment was continued for 48 hours. The spent culture medium was then removed, and the cells were washed once with phosphate-buffered saline (PBS). Fifty micro-litres of 8 mM glucose solution (Roswell Park Memorial Institute medium [RPMI] diluted with PBS and supplemented with bovine serum albumin [BSA] to a final concentration of 0.1%) was added. For the positive control, 1 000 ng/mL insulin were used. The plates were returned to the incubator. After 2 hours, 5 µL culture medium were transferred to a

new 96-well plate and 200 μ L glucose assay reagent (glucose oxidase/peroxidase colorimetric reagent) were added, and the plate was incubated again at 37 °C for 10-20 minutes. The absorbance was measured at 510 nm using the BioTek® SYNERGY Mx fluorometer (Winooski, VT, USA). Glucose utilisation was calculated as the difference between the mean of the no-cell wells and the test sample and expressed as a percentage of the untreated control.

2.3.4 Cytotoxicity evaluation Assay

At the end of the glucose utilisation assay, all the remaining medium was removed. Hundred microliters of fix solution (0.4% formaldehyde in PBS) were added to each well and the plate was incubated at room temperature for one hour. After the removal of the fix solution, 100 μ L of crystal violet (0.1%) were added and the plate was incubated at room temperature again for 20 minutes. The plates were washed with water to remove excess unbound dye and plates were then dried overnight at 37 °C.

Hundred microliters (100 μ L) of acetic acid were as added to each well and the absorbance was read at 570 nm using the BioTek® SYNERGY Mx fluorometer (Winooski, VT, USA). The cytotoxicity is expressed as a percentage of the untreated control.

2.4 RESULTS

The results of the experiment are recorded as Figures 2.1 to 2.10. The overall cytotoxicity test was weak and is unlikely to have any meaningful physiological impact (Figures 2.1 to 2.10). The extracts did not induce glucose utilization greater than activity induced by the controls: insulin and metformin.

2.4.1 The Experimental results of Glucose utilization bioactivity testing of five *Medicago sativa* L. leaf extracts in C3A/HEPG2 liver cell.

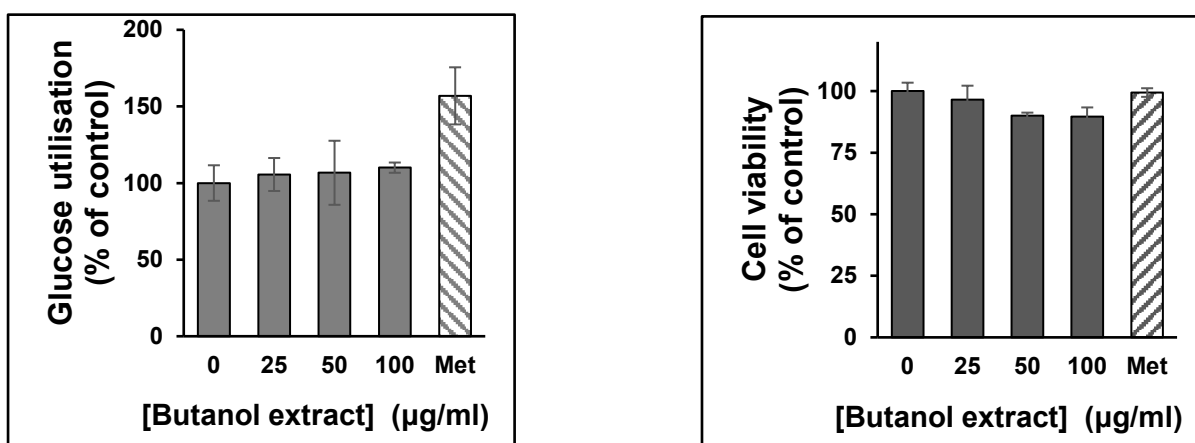


Fig. 2.1 The Graph of Glucose-Utilization and Cell viability (cytotoxicity) induced by the Butanol extracts (four different concentrations) of *Medicago sativa* L. in C3A/HEPG2 liver cells, Metformin served as our control. All four concentrations of plant extracts induced a consistent glucose utilization and cytotoxicity compared to metformin.

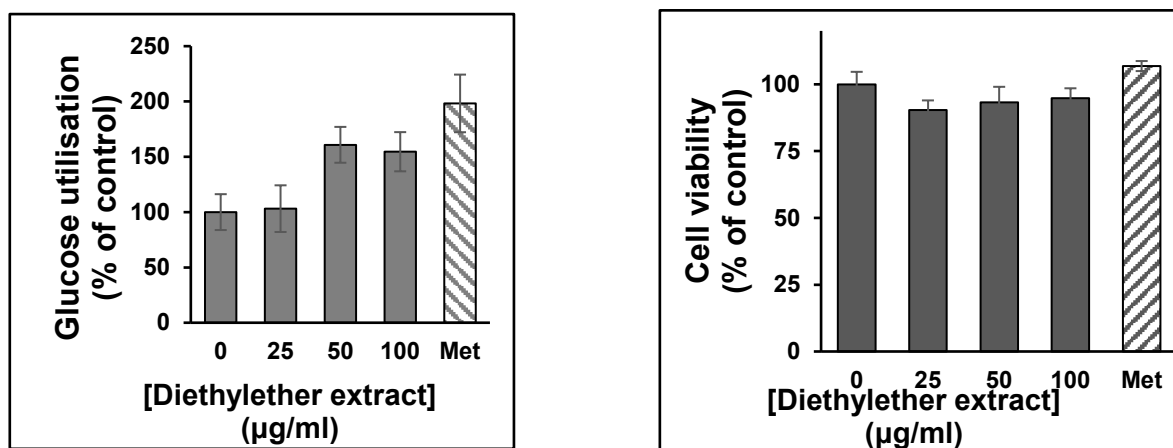


Fig. 2.2 The Graph of Glucose-Utilization and Cell viability (cytotoxicity) induced by the Diethyl-ether extracts of *Medicago sativa* L. in C3A/HEPG2 liver cells, Metformin served as our control. 50 µg/ml and 100 µg/ml of the Diethyl-ether extracts exhibited better glucose utilization activity that is not bad compared to metformin.

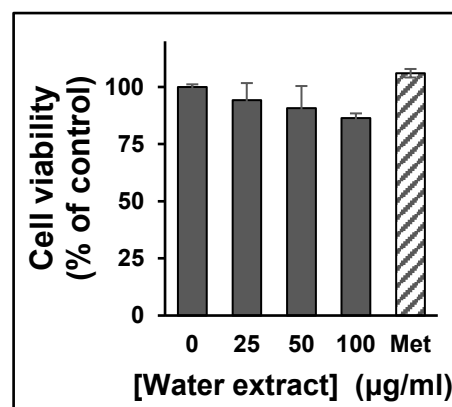
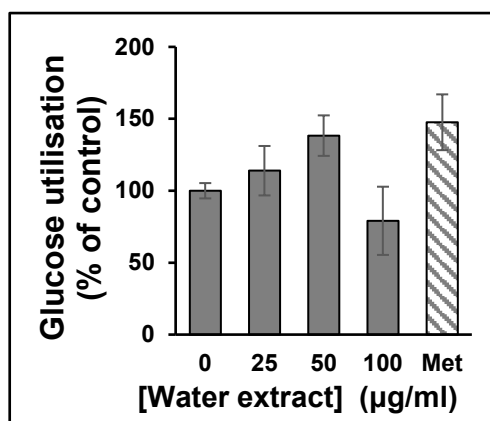


Fig. 2.3 The Graph of Glucose-Utilization and Cell viability (cytotoxicity) induced by the Water/Aqueous extracts (different concentrations) of *Medicago sativa* L. in C3A/HEPG2 liver cells, Metformin served as our control. 50 µg/ml of the water extract shows a better glucose-utilization bioactivity.

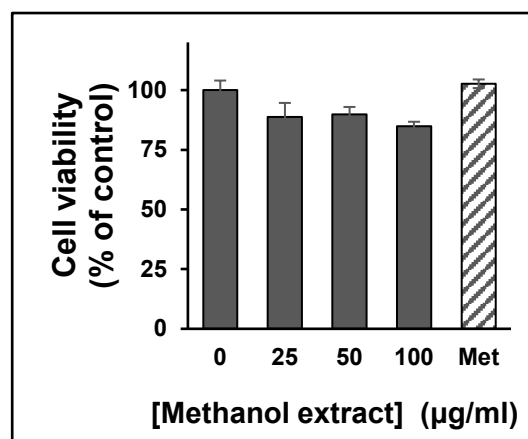
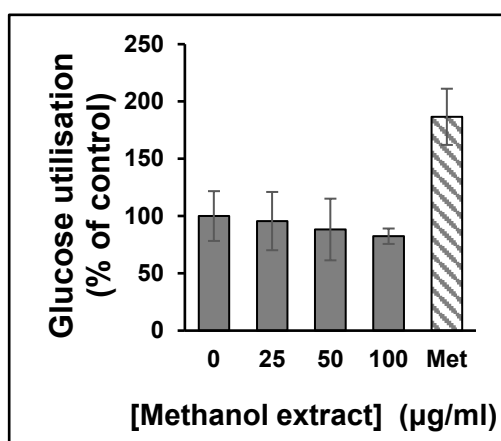


Fig. 2.4 The Graph of Glucose-Utilization and Cell viability (cytotoxicity) induced by the Methanol extracts of *Medicago sativa* L. in C3A/HEPG2 liver cells, Metformin served as our control. The tested methanol extracts produced half of the glucose utilization activity induced by our control, metformin.

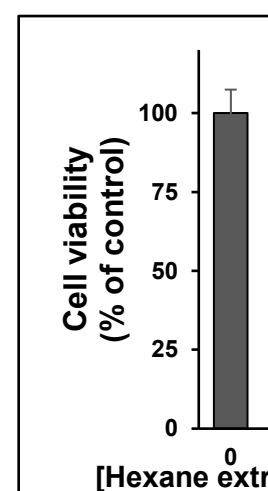
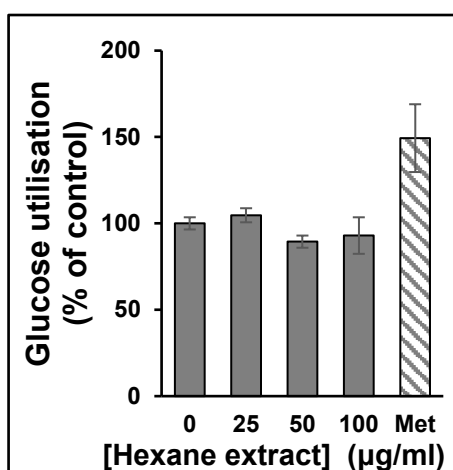


Fig. 2.5 The Graph of Glucose-Utilization and Cell viability (cytotoxicity) induced by the Hexane extracts of *Medicago sativa* L. in C3A/HEPG2 liver cells, Metformin served as our control. The tested Hexane extracts produced considerable glucose utilization activity relative to our control, metformin. The cell viability induced by our extracts is consistent with the cytotoxicity activity of the y our extracts is consistent with the cytotoxicity activity of the control.

2.4.2 The Experimental results of Glucose utilization bioactivity testing of five *Medicago sativa* L. leaf extracts in L6 mouse skeletal muscles cells.

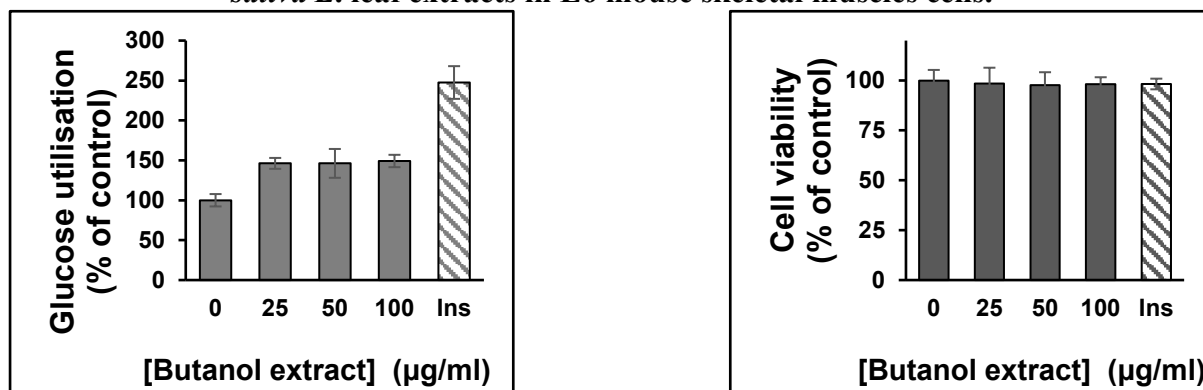


Fig. 2.6 The Graph of Glucose-Utilization and Cell viability (cytotoxicity) induced by the Butanol extracts (different concentrations) of *Medicago sativa* L. in L6 Mouse Skeletal cells, Insulin served as our control. The tested plant extracts induced glucose use that is more than 50 percent of the bioactivity induced by insulin.

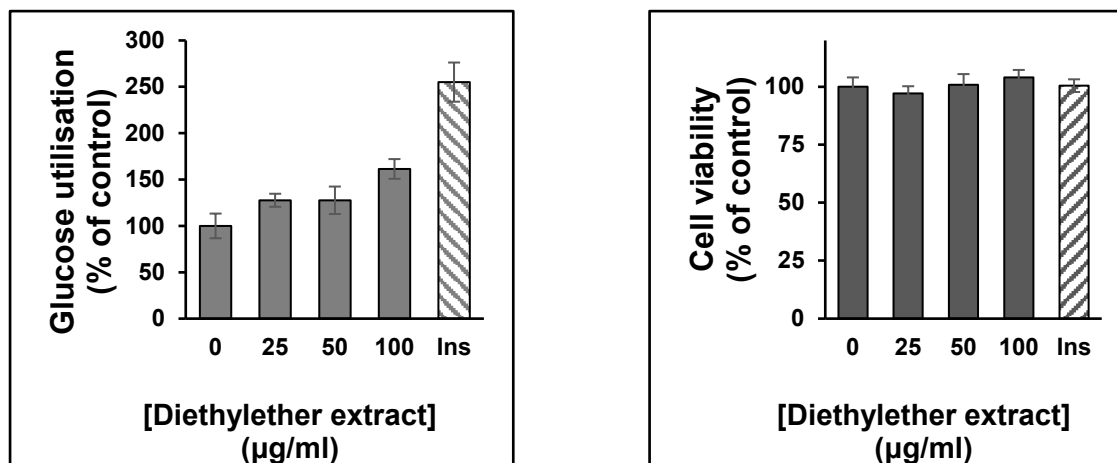


Fig. 2.7 The Graph of Glucose-Utilization and Cell viability (cytotoxicity) induced by the Diethyl-ether extracts (different concentrations) of *Medicago sativa* L. in L6 Mouse Skeletal cells, Insulin served as our control. The plant extract induced dose-dependent glucose use.

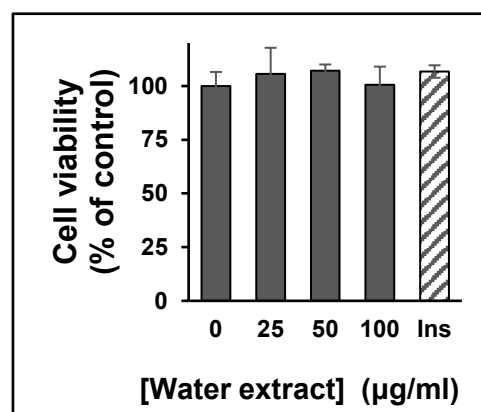
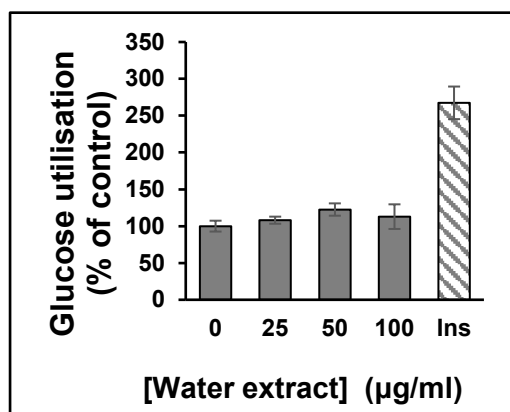


Fig. 2.8 The Graph of Glucose-Utilization and Cell viability (cytotoxicity) induced by the Water/Aqueous extracts (different concentrations) of *Medicago sativa* L. in L6 Mouse Skeletal cells, Insulin served as our control. The water extract in L6 exhibited a poorer glucose use compared to the water extract in C3A/HEP cells.

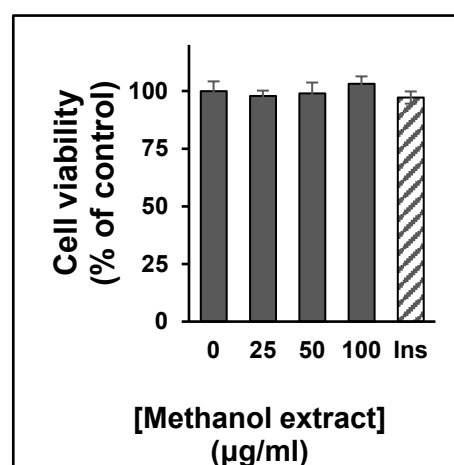
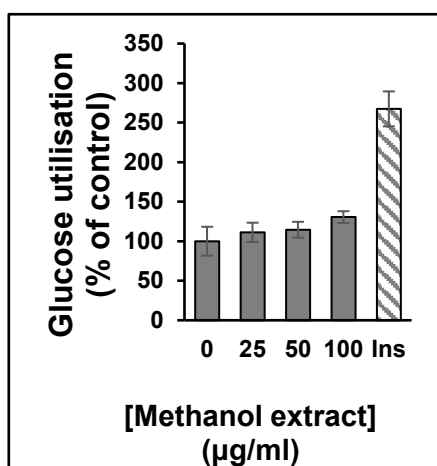


Fig. 2.9 The Graph of Glucose-Utilization and Cell viability (cytotoxicity) induced by the Methanol extracts (different concentrations) of *Medicago sativa* L. in L6 Mouse Skeletal cells, Insulin served as our control. The Methanol extracts did not produce a significant Glucose use in the L6 Skeletal Muscles cell as well as in the liver cells (Fig. 2.4).

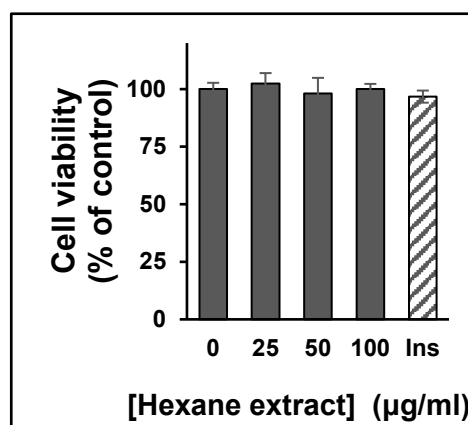
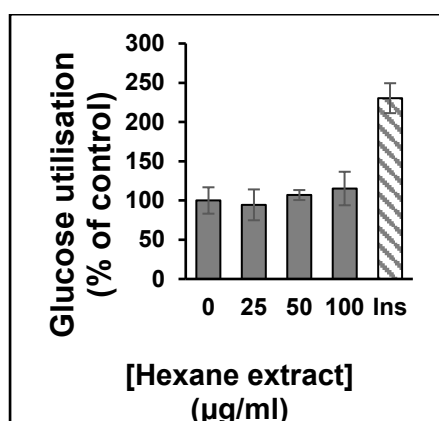


Fig. 2.10 The Graph of Glucose-Utilization and Cell viability (cytotoxicity) induced by the Hexane extracts (different concentrations) of *Medicago sativa* L. in L6 Mouse Skeletal cells, Insulin served as our control. The hexane extracts performed better in the liver cell line (HEPG2/C3A) than in the L6 cells. The all extracts of Alfalfa produced considerable Glucose-utilization bioactivity, which confirms that Alfalfa tea can be used as a natural preventative medicine towards DM.

2.5 DISCUSSION AND CONCLUSION

The objective of our experiments was to measure and report the in-vitro glucose utilization activity induced by five extracts of alfalfa and compare their activity to clinical drugs: insulin and metformin. The Diethyl-ether (50 µg/mL and 100 µg/mL) and water extracts (50 µg/mL) of alfalfa induced considerable increase in glucose utilisation towards the hepatic HEPG2/C3A cell line (Figures 2.2 and 2.3) favourable to the bioactivity exhibited by the positive control metformin. The liver is the central site of glucose control by regulating the uptake and storage of glucose (Nordlie et al., 1999). For experiments we used metformin as a control. Metformin is a nature-derived therapeutic medicine that is widely used clinically to treat Type 2 diabetes (Rena et al., 2017). Metformin is derived from the plant *Galego officinalis*, which is famously known as Goat's rue, a plant of medicinal importance that was widely used for diabetes and breast-feeding in medieval Europe (Rena et al., 2017). Although metformin does not induce hypoglycaemia and weight gain, there are incidences where the patients presented with lactic acidosis (Stein et al., 2013). The compound metformin works by increasing insulin sensitivity by inducing increased uptake and utilization of glucose, by lowering hepatic gluconeogenesis and by decreasing intestinal absorption of glucose (Rena et al., 2017). The exhibited glucose utilization biological activity by alfalfa leaf extracts validates the ethnobotanical claims that the herbal infusion made with dried alfalfa leaf induces beneficial anti-diabetic effects (Liu et al., 2018). Further experimentation and active-compound identification are justly recommended.

The polypeptide hormone insulin that is produced by beta cells in the pancreas plays a central role in diabetes. The decreased activity or decreased levels of insulin causes high blood glucose levels which is called DM (Friedman et al., 1992). The functioning of insulin is important in that it allows the decrease of blood glucose by allowing muscle and fat cells to take up glucose through the help of GLUT-4. The take-up of glucose by fat cells yields a production of triglycerides, which explains why insulin treatment often causes undesired weight gain in diabetic patients with high insulin resistance (Friedman et al., 1992). Human insulin (or insulin human) is a drug synthesized through DNA-recombinant techniques to control and regulate the rate of glucose and amino acid cellular uptake. Insulin is injected subcutaneously to affect the blood glucose levels in Type 1 and Type 2 DM. the common side effects of insulin therapy include excessive hypoglycaemia, weight gain, gut discomfort, nausea, and the usual organ failure (liver and heart).

The results demonstrated that all the five extracts tested exhibited considerable glucose utilization enhancement both at the liver and skeletal muscle level, although not strong enough activity relative to the clinical controls used (metformin and insulin). The experimental results correlates with the therapeutic reports alluded to the religious drinking of alfalfa tea for health and wellness. *Medicago sativa L.* appears in ancient medicinal herbs/plant books like the Compendium of *Materia Medica* by Li Shizhen, and it is in there described as the father of all foods: *alfac-facah* (Liu et al., 2018) and our experimental results can be used to scientifically validate that opinion. Future studies may determine in-vivo testing to establish the possible mechanisms of action exhibited by alfalfa plant.

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CHAPTER III-EXPERIMENT II:

DPPH SCAVENGING, AND ANTI-INFLAMMATORY TESTING OF ALFALFA



One of the edible plants grown from seed by the candidate Thapelo Makhele. Lifestyle change is expensive, but if you have access to sunshine and water, organics, and seeds then you can continually reap what you sow. Tomatoes are reported to be rich sources of antioxidants but here they were grown for breakfast on a budget.

3.1 ABSTRACT

***IN-VITRO* DPPH SCAVENGING ACTION, AND THE ANTI-INFLAMMATORY BIOACTIVITY TESTING OF FIVE *Medicago sativa* L. (Alfalfa) Leaf EXTRACTS USING THE MOUSE MACROPHAGE CELL LINE RAW 264.7.**

Makhele Thapelo Simon, Makhoahle Pakiso Moses, and Mashele Sitheni Samson

Oxidative stress, inflammation and hyperglycaemia were determined as key players in the Corona Disease of 2019 (COVID-19) (Beltran-Gracia et al., 2020). Although many COVID-19 patients were largely asymptomatic and only a few were moderately symptomatic; about five percent (5%) of them presented with hyperglycaemia, perfusion injury, septic shock and sometimes with multiple organ failure (Beltran-Gracia et al., 2020). The value of antioxidants in disease prevention and management, is in the alleviation of oxidative stress through free radical scavenging and decomposing actions (Khalil et al., 2019). Low-grade chronic inflammation and oxidative stress is implicated in diseases that include cancer, cardiovascular diseases, neurodegenerative diseases and ageing, liver, and kidney disease. The modern lifestyle is bloated by higher consumption of processed foods, increased exposure to environmental and industrial toxins and pollutants, and the lack of physical activity (sedentary lifestyle), all of which increase the prevalence of oxidative stress and chronic inflammation. Natural and medicinal plants contain compounds that can be extracted and purified for usage as therapeutics, antioxidants, and anti-inflammatories with lesser negative side effects compared to clinical and over-the-counter synthetic medicines. To measure the antioxidant activity induced by five extracts of *Medicago sativa* L. leaves, the DPPH Radical Scavenging Assay was performed. To evaluate the anti-inflammatory bioactivity exerted by the same five extracts of *Medicago sativa* L. (Alfalfa) leaf, an anti-inflammatory assay of extracts was performed using the mouse (muscarine) macrophage cell line RAW 264.7. The reliable EGCG and Trolox served as our controls. To confirm the absence of cytotoxicity as a contributory factor, cell viability was also assessed using an Methylthiazolyldiphenyl-Tetrazolium Bromide (MTT) assay. From our experimental results, the water extract exerted greater DPPH scavenging activity. All the other extracts of Alfalfa induced relatively poor DPPH scavenging activity, except for the butanol and methanol extracts at 400 µg/ml. Five extracts were tested for anti-inflammatory activity as well as their corresponding toxicity using RAW 264.7 macrophages. Aminoguanidine (AG) was used as a positive control.

The only sample to show promising anti-inflammatory activity is the hexane extract. None of our tested samples exhibited considerable cytotoxicity. Although inflammation is usually associated with a protective or healing response, many chronic diseases are characterised by persistent inflammation ultimately resulting in tissue dysfunction (Lee and Yang, 2013). The aqueous, butanol, methanol and hexane extracts of *Medicago Sativa L.* leaves exhibited some antioxidant and anti-inflammatory activities respectively, as thus potentiating Alfac-facah tea use to be beneficial for maintaining health and wellness.

Keywords: Reactive Oxygen Species (ROS). Free Radicals. Oxidative Stress. Low grade Chronic Inflammation. Cardiovascular Diseases (CVD). Antioxidants. *Medicago sativa L.* (Alfalfa/ Lucerne). Muscarine/ Mouse Macrophage Cell Line RAW 264.7. Trolox. Epigallocatechin gallate (EGCG). 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT). Lipopolysaccharide (LPS), Nonsteroidal anti-inflammatory drug (NSAID). 2,2-diphenyl-1-picrylhydrazyl (DPPH). Corona Disease of 2019 (COVID-19)

3.2 BACKGROUND

The excess accumulation of pro-oxidants beyond oxidative-homeostatic control and regulation plays a key role in the pathogenesis of many chronic diseases (Sharif-Rad et al., 2020). Low-grade chronic inflammation and oxidative stress is implicated in diseases that include cancer, cardiovascular diseases, neurodegenerative diseases and ageing, liver, and kidney diseases (Biswas, 2016). The modern lifestyle is bloated with the consumption of processed foods and fast foods (cooked using high heat, vegetable oils, hydrogenated oils and salty food additives like spices and sauces), increased exposure to environmental and industrial toxins and pollutants, and the lack of physical activity, all of which are factors that increase the prevalence of oxidative stress (Sharif-Rad et al., 2020). There are many negative implications of persistent oxidative stress such as low-grade chronic inflammation, cellular damage, steroid resistance, necrosis, epigenetic changes, autoimmunity and many more (Aggarwal et al., 2018). The balance between pro-oxidants and antioxidants is critical for the correct physiologic functioning of bodily systems (Checconi et al., 2020). Antioxidants are molecules that interact with free radicals and as thus terminating the adverse chain reactions and converting them to harmless products (Khalil et al., 2019). The value of antioxidants in disease prevention and management lies in the alleviation of oxidative stress through free radical scavenging and decomposing functions (Khalil et al., 2019). There are reports in the

literature of adverse reactions from synthetic antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole (Lobo et al., 2010).

According to the World Health Organization (WHO), the biggest killer in the world is Ischaemic Heart Disease, which accounted for 8.9 million deaths in the year 2019 (WHO 2020). The risk factors of Ischaemic Heart Disease include hypertension and hypercholesterolemia, which then contribute to the total generation of ROS and as thus induce oxidative stress (Victor et al., 2017). The low levels of ROS in the system can provide essential benefits such as endogenous cardioprotective actions, anti-atherosclerotic and pro-angiogenesis protection (Taverne et al., 2013). The health challenges with high ROS in cardiovascular diseases (CVD) include endothelial dysfunction, myocardial ischemia/reperfusion injury, heart failure and cardiac arrhythmias (Taverne et al., 2013; Sharifi-Rad et al., 2020). ROS are the resultant by-products of cellular metabolism that can induce the inactivation of signalling mechanisms between insulin receptors and the glucose transport system and thus induce insulin resistance (Sharifi-Rad et al., 2020).

In diabetes, an electron transfer and oxidative phosphorylation are decoupled, resulting in the production of superoxide anions and inefficient ATP synthesis (Sharifi-Rad et al., 2020). Insulin Resistance (IR), abdominal obesity, atherogenic dyslipidaemia, high blood pressure, genetic predisposition and chronic stress are the main risk factors underlying the metabolic syndrome (Sharifi-Rad et al., 2020).

Diabetic cardiomyopathy presents clinically with left ventricular hypertrophy, myocardial fibrosis, and impaired diastolic function. Hyperglycaemia has been demonstrated to facilitate oxidative stress and also to promote excessive ROS production, both of which can be implicated in the observed cardiovascular damages in diabetic complications. ROS has been demonstrated to cause myocardial growth, and cellular dysfunction by activating several hypertrophic signal kinases and transcription factors. However, ROS also stimulates apoptosis signal kinase-1: a very sensitive redox kinase that often leads to NF- κ B-induced hypertrophy (Guzik and Harrison, 2007). Heart Failure (HF) is a progressive disease with the yearly mortality rate of 10%. Mitochondrial ROS and mitochondrial matrix calcium have since been shown to participate in the pathogenesis of obesity-induced heart failure where they attack cardiomyocytes through mechanisms that involve free radical injury and inflammatory cytokines, which results in the observed cardiomyocytes apoptosis, decreased cardiac

function and myocardial hypertrophy (Niemann et al., 2020; Das et al., 2012; Zeng et al., 2019).

Aloe produces an exudate that contains many compounds, including aloe-emodin (1,8-dihydroxy-3-hydroxymethyl), which were demonstrated to prevent myocardial infarction through upregulating the miR-133, thereby inhibiting the caspase-3 apoptosis signal pathway in a H₂O₂-induced apoptosis model of neonatal rat ventricular myocytes (Yu et al., 2019). Furthermore, aloe-emodin was established experimentally to reduce cardiac inflammation that is induced by high-fat diet through the TLR4 signalling pathway by Chen et al., 2020. Neurodegenerative Disorders (NDDs) affect millions of people worldwide, causing sensor-motor impairment. The neuroprotective properties of tea polyphenols have been demonstrated in many studies, for example the beneficial therapeutic activities of the hydroxyl groups of tea catechins EGCG (epigallocatechin gallate), where ring B and D allow them to interact with free radicals. For example, Zhao et al. (2014) confirm that “Green tea polyphenols protected spinal cord neurons from damage caused by hydrogen peroxide-induced oxidative stress”. This study and many others confirm that the consumption of Alfalfa tea mixed with green tea can be used as a non-drug-based therapeutic agent.

Dan-Dan Zhang and colleagues of the Shanghai University of Traditional Medicine in China demonstrated the mechanism of action of 1, 3, 5, 7-Tetrahydroxy-8-isoprenylxanthone (TIE) isolated from a species of *Garcinia Linn.* (Dan-Dan Zhang et al., 2015). The species of *Garcinia Linn.* are proven to be rich in natural xanthenes and benzophenones, both which bless the drug development industry with compounds that are both structurally diverse and those that impose a wide variety of proven pharmacological activities. In their well-established experiment, they established both the anti-inflammatory activity and the mechanism of action; where the extracted compounds inhibited the production of nitric oxide and prostaglandin E₂ (PGE₂) in LPS/IFN-gamma-stimulated RAW264.7 cells. Furthermore, they went to show that TIE suppressed the expression of inflammatory cytokines IL-6, IL-12, and TNF-alpha; molecules that are notoriously implicated in obesity, whereby they are secreted by adipocytes which then causes the resulting inflammatory state. Woan Sean Tan and colleagues of the Laboratory of Vaccines and Immunotherapeutics, based at the University of Putra Malaysia, also demonstrated the suppression of the activation of the inflammatory mediators in LPS-stimulated RAW264.7 macrophages via the nuclear factor-kappa B (NF-kB) by the hydroethanolic extract of *Moringa oleifera*. Hamza Mechachate of the Laboratory of Biotechnology at the University of Sidi Mohamed Ben Abdellah

(USMBA), with collaborators based in Brazil and Finland, also demonstrate in-vitro and also in mice the anti-inflammatory effects of the Polyphenol-Enriched Fraction Extract of *Myrtus communis* L leaves.

The old hunter-gatherer was blessed with access to natural compounds that are both medicinal and nutrient rich. The relationship between the African hunter and his surroundings included the ability to communicate with the honeybee (*Apis mellifera scutellata*), which guided him towards the beehive hidden deep in the bushy African forest. Propolis is a resinous compound produced by honeybees that is rich in pharmacological-active compounds that include the anti-carcinogenic caffeic acid phenethyl ester (CAPE), artemisinin, and chrysin. The bioactivity of Propolis is towards multiple signalling pathways, including phosphoinositide 3-kinase (PI3K)/Akt and to induce cell cycle arrest. The aim of this experimental chapter was to establish for the first time the antioxidant scavenging activity, as well as to determine the anti-inflammatory potential of five extracts of *Medicago sativa* L leaves.

3.3 MATERIALS AND METHODS

3.3.1 The Test (Plant Material), The Extraction and The Preparation for in-vitro Bio-Analysis

The plant materials (Figure 1) were sourced north of Pretoria (in South Africa) with the Batch Number MH 71(10 kg). The material was air-dried and stored in a cool dry area away from light and heat. The dry, grassy, and pale green leaves were extracted using five solvents: butanol, diethyl-ether, hexane, methanol, and water. Sixty (60) grams of powdered plant material were extracted in 1 litre (1 000 mL) of every solvent. The extracts were filtered using the Buchner funnel and Whatman no.1 filter paper. The extracts were frozen at -40 °C and freeze-dried for 48 hours at a yield of 9 g of dried extracts. The dried extracts were stored at -4 °C until analysis.

3.3.2 The DPPH Scavenging Assay, The Anti-Inflammatory, and Cell Viability (MTT) Assay

3.3.2.1 Material

Positive control stocks: Trolox (a water-soluble analog of vitamin E, chosen for its potency towards oxidative stress) and Epigallocatechin gallate (EGCG, which is a plant based

catechin or polyphenol that is confirmed to be potent protector of cellular damage due to oxidative stress) were sourced from Sigma. DPPH: 2,2-diphenyl-1-picrylhydrazyl also from Sigma. Ethanol 99.9% (Sigma). Tris (Roche Diagnostics GmbH, Mannheim, Germany). RAW 264.7 mouse macrophages were purchased from Cellonex (South Africa). Lipopolysaccharide (LPS), Greiss reagent and Aminoguanidine (AG) were purchased from Sigma-Aldrich (St. Louise, MO, USA)

3.3.2.2 Assay Procedure of the DPPH Scavenging Activity

The reagents required for the DPPH assay were prepared according to Table.1: Tris-HCl buffer (50 mM): we dissolved 0.606 g Tris (Roche Diagnostics GmbH, Mannheim, Germany) in 80 ml of distilled water; adjusted the pH to 7.4 using HCl then filled up to 100 ml with distilled water. DPPH (0.1 mM): we dissolved 0.002 g of DPPH in 50 ml ethanol; prepared fresh and protected from light. Extracts were tested at a range of 6.25 – 400 µg/ml. Assay was performed in triplicate.

Absorbance was measured using a Biotek® Powerwave XS microtiter plate reader (Winooski, VT, USA). The Percentage of antioxidant activity (AA%) was calculated using the formula below:

$$AA\% = 100 - \left[\frac{(Abs_{sample} - Abs_{blank}) \times 100}{Abs_{control}} \right]$$

❖ Sample	5 µL
❖ Tris-HCl (50 mM, pH 7.4)	120 µL
❖ DPPH (0.1 mM)	120 µL
Incubate the 96-well plate for 20 minutes at room temperature	
Measure absorbance at 513 nm	

Table 3.1 sample Volumes and preparations for the 96-well plate assay

3.3.2.3 The Anti-Inflammatory and Cell Viability (MTT) Assay

Sample preparation: Extracts were solubilised in DMSO to a final concentration of 100 mg./ml and further diluted into culture medium. Aminoguanidine (AG) was used as a positive control to indicate anti-inflammatory activity.

Anti-inflammatory screening protocol: RAW 264.7 cells were seeded into 96-well plates at a density of 1×10^5 cells per well and allowed to attach overnight. The following day spent culture medium was removed and the samples (samples were diluted in DMEM complete medium) were added to give final concentrations of 25, 50, 100 and 200 $\mu\text{g/mL}$ (50 μl per well at double the desired final concentration). To assess the anti-inflammatory activity, 50 μL of LPS (final concentration of 500 ng/mL) containing medium were added to the corresponding wells. Aminoguanidine (AG) was used as the positive control for this experiment. Cells were incubated for a further 18 hours. To quantify NO production, 50 μL of the spent culture medium were transferred to a new 96-well plate and 50 μL Griess reagent added. Absorbance was measured at 540 nm, and the results expressed relative to the appropriate untreated control. A standard curve using sodium nitrite dissolved in culture medium was used to determine the concentration of NO in each sample. Assay was performed in triplicate.

3.3.2.4 Cytotoxicity (Cell Viability) screening protocol

To confirm the absence of toxicity as a contributory factor, cell viability was assessed using MTT assay. This was done by the removal of the remaining of the medium and treatments in each well and replacing it with medium containing 0.5 mg/mL MTT and further incubated for 30 minutes at 37 °C. Thereafter, MTT was removed and 200 μL DMSO were added to each well to solubilise the formazan crystals. Assay was performed in triplicate.

Absorbance was read at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA).

3.4 RESULTS

3.4.1 The experimental results of the DPPH Scavenging Activity, and Anti-Inflammatory Potential Screening of five extracts of *Medicago sativa L.* leaves (Solvents: Butanol, Diethyl-ether, Hexane, Methanol and Water)

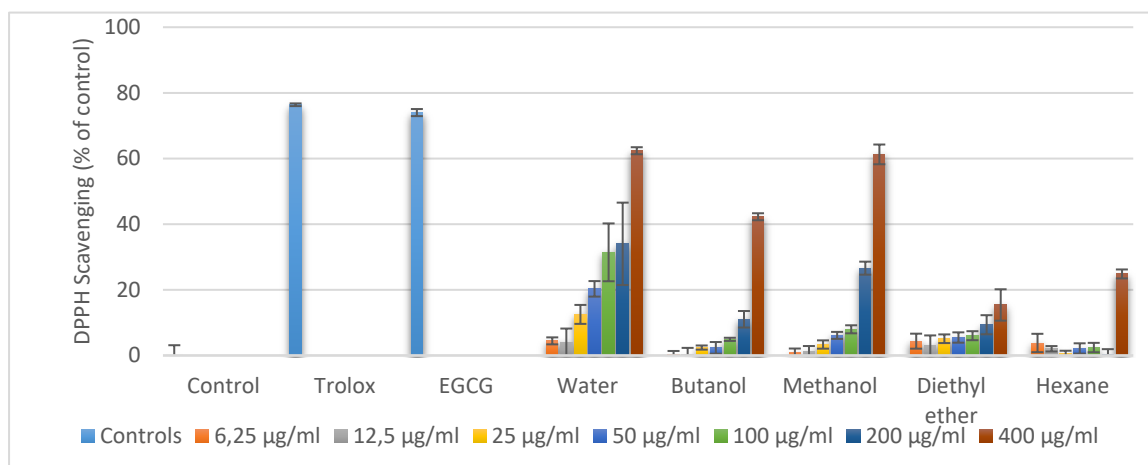


Fig. 3.1 The graph of DPPH scavenging potential testing of Alfalfa leaf extracts. Trolox and EGCG served as positive controls at 100 μ M. Error bars represent standard deviation of quadruplicate values. Five extracts were compared at varied dosages. Water and Methanol extracts at the dosage of 400 micro-grams per millilitre, exhibited significant DPPH scavenging action relative to controls (62.3 % and 61.2 %). Trolox and EGCG exhibited more or less the same activity (76.38% and 73.99%). Herbal tea is the aqueous extraction that people use to siphon phyto-nutrients and therapeutic compounds contained in the herbs, trees, leaves, roots, and seeds.

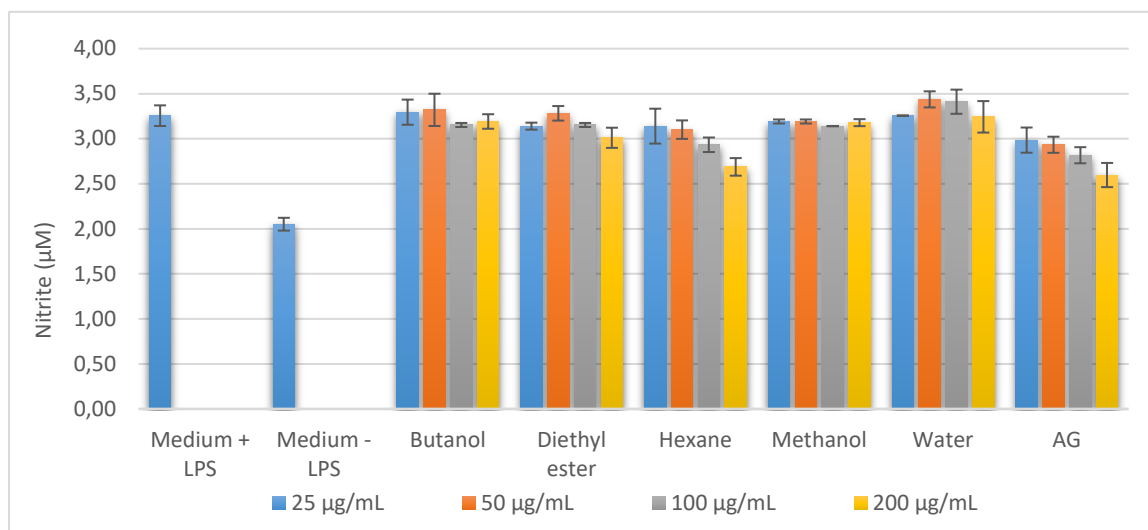


Fig. 3.2 The graph of Nitric oxide production in LPS activated macrophages treated with different concentrations of Alfalfa extracts and the control Aminoguanidine (AG). Bar graph represents triplicate values of one experiment. Error bars represents the standard deviation of the mean. Aminoguanidine (AG) is a potent scavenger of ROS and has been developed to prevent inflammation, and renoprotective effects in Renal Ischaemia and Reperfusion Injury (IRI).

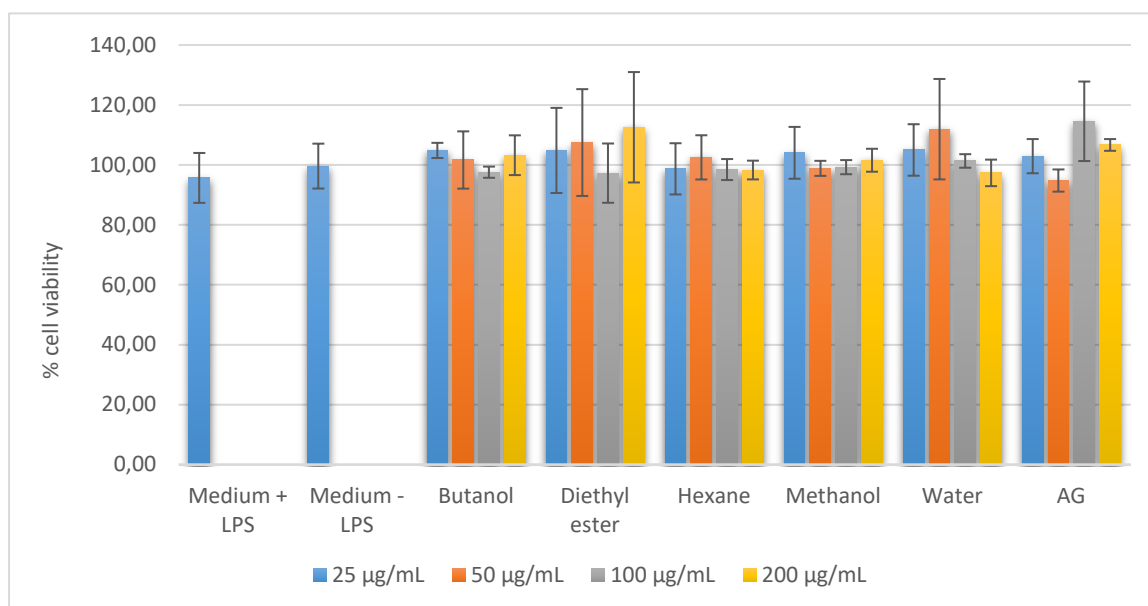


Fig. 3.3 The graph of: Cell viability (%) of LPS activated macrophages after 24 hours of exposure to treatments. Bar graph represents triplicate values of one experiment. Error bars represents the standard deviation of the mean. Aminoguanidine (AG) is known to exhibit oxidative stress and inflammation inhibition. All the tested Alfalfa extract dosages did not induce worrying cytotoxicity.

3.5 DISCUSSION AND CONCLUSION

DPPH (2,2 - diphenyl-1-picryl-hydrazyl-hydrate) free radical scavenging method is an antioxidant assay based on electron-transfer that produces a violet solution in ethanol. This free radical, which is stable at room temperature, is reduced in the presence of an antioxidant molecule, thus giving a colourless ethanol solution which is measured at 513 nm using a Biotek® Powerwave XS microtiter plate reader (Winooski, VT, USA). The experimental results show that the water extract had the best DPPH scavenging activity, which indicates that it has the best therapeutic potential towards the relief of oxidative stress and low-grade chronic inflammation (Figure 3.1). All the other extracts we tested had relatively poor DPPH scavenging activity, except for the butanol and methanol at 400 µg/mL (Figure 3.1). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a Vitamin E analogue that is indicated for the reduction of oxidative stress and consequent oxidative-stress damage. Trolox plays an essential role in antioxidant bioactivity testing such as in the Trolox Equivalent Antioxidant Capacity Assay (TEAC) and the Ferric Reducing Ability of Plasma (FRAP) Antioxidant Capacity Assay.

Although inflammation is usually associated with a protective or healing response, many chronic diseases are characterised by persistent inflammation ultimately resulting in tissue

dysfunction (Lee et al., 2013). Macrophages perform a variety of complex microbicidal functions, including surveillance, chemotaxis, phagocytosis, and destruction of pathogenic micro-organisms (Ahn et al., 2015). Therefore, the development of therapeutics which can non-specifically augment the innate immune response represents a promising strategy to combat classical and emerging infectious agents. Five extracts (at 25, 50, 100, 200 $\mu\text{g/mL}$) were tested for anti-inflammatory activity as well as their corresponding toxicity using RAW 264.7 macrophages cell line (further experimentation at 400 $\mu\text{g/mL}$ is recommended because of promising DPPH activity exhibited by the 400 $\mu\text{g/mL}$ tests concentration. Figure 3.2 shows the results of the experiment, Trolox and EGCG was used as a positive control. The water, and methanol extracts of lucerne at 400 $\mu\text{g/mL}$ exhibited promising DPPH scavenging activity relative to the controls Trolox and EGCG (Figure 3.2). The mouse macrophage cell line, RAW 264.7, is a well characterised and popular model to investigate the anti-inflammatory potential of test samples. Cells are cultured in multi-well plates and activated by exposure to LPS, which induces the expression of iNOS with concomitant nitric oxide formation. Changes in NO production are determined by measuring the levels of nitrate in the culture medium. Simultaneous evaluation of cell viability (MTT assay) is used to confirm the absence of cytotoxicity of the test sample. No samples showed any cytotoxicity following the treatments (Figure 3.3).

The urban lifestyle is fast, and as a result, fast processed foods are in great demand. This modern approach to nutrition, coupled with environmental (cellular radiation) and industrial toxin exposure increases the prevalence of oxidative stress and chronic inflammation (Sharifi-Rad et al., 2020). Due to the established negative implications of oxidative stress and chronic inflammation in human diseases, non-drug alternatives are more preferred due to fewer side effects they cause. The consumption of tea as a health-enhancing beverage predates many modern medical interventions in disease prevention and treatment. *Pitsa* is a Sesotho name that refers to a concoction of medicinal plants for the purpose of disease prevention, management, and cure. Our experiments confirm the existing knowledge that *Pitsa* exhibits potent pharmacological bioactivity against infections, NCDs, and as well as a general reviver of vitality. In our experiments, *Medicago Sativa* L. experimentally exhibited some antioxidant and anti-inflammatory bioactivity, and no samples of *Medicago Sativa* L. showed any cytotoxicity. The results of our experiments correlate with the ethno-medicinal uses of Alfalfa, where all types of ruminants are fed fodder containing Alfalfa and humans claim the therapeutic benefits of the seed and leaves of *Medicago sativa* L. We therefore

recommend further experimentation with 400 µg/mL extracts towards DPPH scavenging activity and in-vivo models to test the therapeutic compounds contained in *Medicago Sativa* L.

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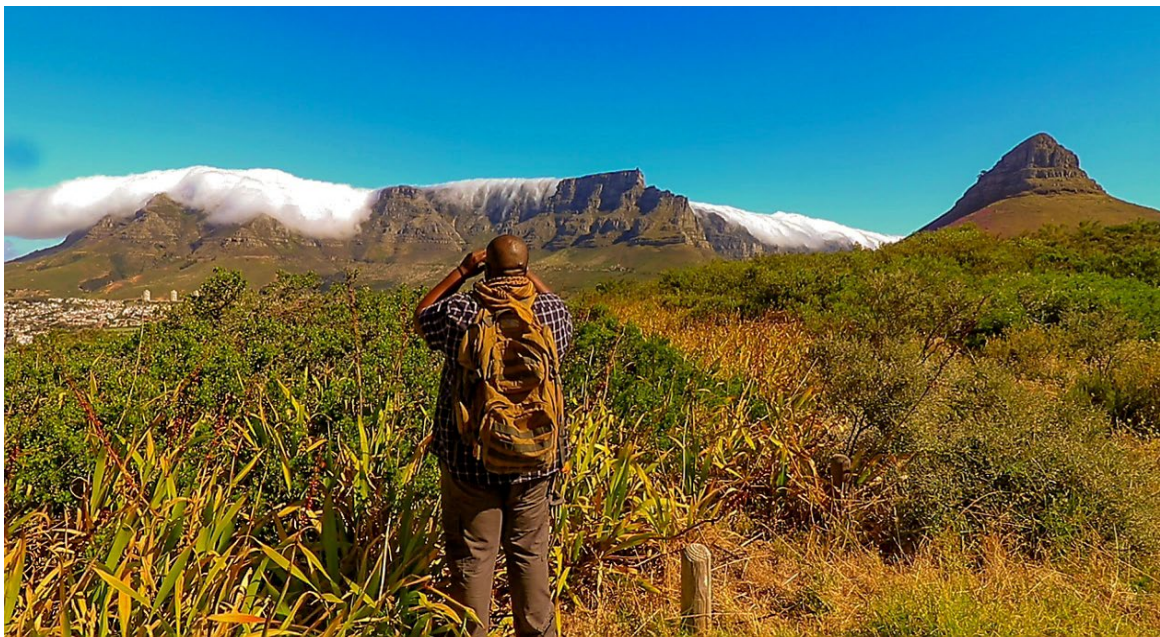
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CHAPTER IV-EXPERIMENT III:

THE IN-VITRO ANTI-CANCER TESTING OF ALFALFA



The Candidate Thapelo Makhele exploring the land where Vasco da Gama and Bartolomeu Dias landed, a biodiverse area with plant species that can only be found there. The white tablecloth appears early in the morning and as a hobbyist photographer it was an interesting natural marvel to self-photograph within.

4.1 ABSTRACT

ANTI-NEOPLASTIC AND CYTOTOXICITY POTENCY MEASURING OF FIVE *Medicago sativa* L. (Alfalfa) LEAF EXTRACTS AGAINST MELANOMA (UACC62), BREAST (MCF7), PROSTATE (PC3), AND COLON (HCT116) CANCER CELLS

Makhele Thapelo Simon, Makhoahle Pakiso Moses, and Mashele Sitheni Samson

The National Cancer Registry of the National Department of Health of South Africa measured that in females, the highest incidence of cancers diagnosed in the year 2019 were breast (10 172 cases), cervical (6 945 cases) and colorectal (1 952 cases) cancers, while in males the most diagnosed cancers were prostate, lung, and colorectal neoplasms. The risk factors that promote cancer development and metastasis include excessive alcohol and narcotic use, sedentary lifestyle, carcinogenic infections, and genetic predispositions. The global incidence of cancer is much likely to increase because of the increased exposure to risk factors that promote oxidative stress, low grade chronic inflammation, diabetes mellitus, hyperglycaemia, and insulin resistance. The growth inhibitory and cytotoxicity activity of alfalfa extracts were measured using the reliable Sulforhodamine B Assay. Our *Alfac-facah* leaf test material was extracted using five solvents: ethanol, methanol, diethyl-ether, acetone, and water. Medicinal plants persist to play a key role in medicine, whereby they not only help with treating or preventing diseases, but they also contribute to the general wellbeing of the patient. For this reason, natural resources remain a pivotal ingredient of novel drug development compounds. The anti-cancer activity screening of medicinal plants is the first step in discovering natural chemotherapeutics. The diethyl-ether and acetone extracts displayed some weak activity towards the breast cancer cell line/MCF-7 (3.4 and 3.5) with IC₅₀ of 67.7 µg/mL and 76.81 µg/mL, respectively. All tested extracts of alfalfa exhibited weak-hazard cytotoxicity. The experimental results correlates with the ethno-pharmacological reports that Alfalfa possess considerable chemo-therapeutic potential.

Keywords: NCI (National Cancer Institute, U.S. Department of Health, and Human Services). CSIR (The Council for Scientific and Industrial Research, South Africa). *Medicago sativa* L. (Alfalfa). Sulforhodamine B Assay (SRB). ECACC (European Collection of Authenticated Cell Cultures, UK). Cancer (malignancy, neoplasm, carcinoma, tumour, sarcoma, leukaemia, lymphoma). AICR (American Institute for Cancer Research).

International Agency for Research on Cancer (IARC). THC (*delta-9 tetrahydrocannabinol*). Rick Simpson oil (RSO).

4.2 BACKGROUND

According to the World Health Organization (WHO), cancer is among the leading causes of death in the world, accounting for 8.2 million deaths in 2012 (WHO, 2020a). The International Agency for Research on Cancer (IARC) estimates that more than 60% of the world's new annual cases occur in Africa, Asia, Central and South America (IARC, 2003). These regions account for approximately 70% of the world's cancer deaths. Even though 30% of cancers can be prevented according to the WHO, the IARC estimates that there were 32.6 million people living with cancer in 2012, and the number of new cancer cases is expected to rise from 14 million in 2012 to 22 million cases over the next two decades (IARC, 2003). The current cancer treatment's efficacy is threatened by physical and emotional side effects experienced by hundreds of cancer patients worldwide. Cancer treatment side effects include chemo brain (memory lapse), peripheral neuropathy, pain, fatigue, lymphedema, infections, infertility and reduced sex drive, nausea, vomiting and many more. In 1862, the American antique collector and dealer Edwin Smith procured the ancient medical scroll (the manual for military surgery also known as (*The secret book of the Physician*) from Egypt. The scroll contained earlier cases of injuries, fractures, wounds, dislocations, and tumours and means to cure/treat them. The ancient medical treatise *Edwin Smith Papyrus* indicates how far back in time humans have been facing the burden of cancer and other medical conditions. The fossilised bone tumours observed on ancient Egyptian mummies also confirm that cancer is an old problem that has now become the second leading cause of death according to the WHO (WHO, 2020a; WHO, 2020b).

Carcinogenesis is documented as a multistep process in which distinct molecular and cellular alterations occur. From the studies of experimentally induced carcinogenesis in rodents, tumour development is considered to consist of several separate, but closely linked stages: tumour initiation, promotion, and progression (Aggarwal and Shishodia, 2006; Agrawal et al., 2008; Hafeez et al., 2012). Blocking agents inhibit carcinogens from reaching the target sites, from undergoing metabolic activation or from subsequently interacting with crucial cellular-macromolecules (for example, DNA, RNA, and proteins), while suppressing agents, on the other hand, inhibit the malignant transformation of initiated cells (Karin and Ben, 2000; Karin et al., 2000). The conventional approach to cancer treatment is based on drugs that

retard the proliferation of abnormal cells (cytostatic) or that can kill the abnormal cells (cytotoxic). The use of plants and their extracts in treatment of diseases dates back to before 460-370 BC when Hippocrates practised the art of healing by the use of plant-based drugs. The medicinal value of plants lies in the contained phytochemical (secondary metabolites) substances that can induce a definite physiological action in the human body.

Natural products from natural resources continue to be used in pharmaceutical preparations either as crude extracts, fractions, pure compounds, or analogous compounds (Goodlet et al., 2020). Traditional medical systems of time past still possess great therapeutic value and many medicinal plants have been used thus far to synthesize novel therapeutic compounds based on indigenous pharmacopoeias. The most bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolic compounds (Scalbert and Williamson, 2000). Chemo-preventive phytochemicals can block or reverse the premalignant stage (initiation and promotion) of the multistep carcinogenesis. They can also halt or at least retard the development and progression of precancerous cells into malignant ones. But plants can also deal with factors that promote oxidative stress, inflammation, and hyperglycaemia, thereby making conditions unconducive for malignant cells to grow and spread (Baskar et al., 2010; Crozier et al., 2006; Liu et al., 2019; Chow et al., 2001). More than 250 population-based studies, including case-control and cohort studies, suggest that people who eat five servings of fruit and vegetables a day have approximately half the risk of developing cancer, particularly cancers of the digestive and respiratory tracts (Aggarwal and Shishodia, 2006; Agrawal et al., 2008; Baróniková et al., 2004).

Shintaro Ishikawa and others demonstrated the B16 melanoma metastasis inhibition *in-vivo* that was induced by the JTT extract (Shintaro et al., 2017). Ishikawa et al. demonstrated the activity by injecting (intravenously) C57BL/6 male mice with B16 melanoma cells (2×10^5 cells) and feeding them food containing 3.0 % JTT. The mice were then sacrificed after 3 weeks (21 days) following treatment, and tumour cell colonies on the surface of the lung were counted to measure pharmacological potency of JTT. The experimental results demonstrated the therapeutic actions of JTT being to block B16 melanoma cancer cells, because mice that were not treated with JTT had more than 50 malignant cell colonies, while those that were treated with JTT had less than 20 colonies of metastatic tumour cells (Shintaro et al., 2017). This experiment supports the fact that some medicinal plants not only help with preventing neoplasm development and metastasis, but they can also help with the effects of cancer treatment.

The therapeutic potency of compounds from plants cannot be denied; evidence has been and is still being published on how medicinal plants continue to provide therapeutic metabolites that are antidiabetic, antimicrobial, hepatoprotective, chemo-preventive, antioxidant, anti-inflammatory, and anti-cancer, among many others. For example, Docetaxel (DTX) is a semisynthetic analogue of Paclitaxel, which is isolated from European yew (*Taxus baccata*) that is well used in clinical chemotherapy (Singh et al., 2017). DTX is approved and used in clinical care against breast cancer, ovarian cancer, and non-small lung cancer. DTX was demonstrated to cause cell cycle arrest by interfering with microtubules functions (Singh et al., 2017). The aim of this chapter was to measure the anti-cancer and cytotoxicity activity potential of *Medicago sativa L.* leaf extracts in Melanoma (UACC62), Breast (MCF7), Prostate (PC3), and Colon (HCT116) cancer cells for the first time.

4.3 MATERIALS AND METHODS

4.3.1 The Plant Material, The Extraction and The Preparation for *in-vitro* anti-cancer and cytotoxicity activity measuring

The plant materials were collected from a nursery and medicinal plant farm located few degrees west of north of Pretoria (South Africa) with the Batch Number MH 71(10 kg). The plant material was cultivated by Zizameleni Farming based in Mamogalieskraal, Northwest Province of South Africa. The test material was cultivated using regenerative natural farming principles and the fertilizers used were all natural and certified organics. The material was air-dried and stored in a cool dry area away from light and heat. The dry, grassy, and pale green leaves were extracted using five solvents: ethanol, methanol, diethyl-ether, acetone, and water. Sixty (60) grams of powdered plant material were extracted in 1 litre (1000 mL) of every solvent. The extracts were filtered using the Buchner funnel and Whatman no.1 filter paper. The extracts were frozen at -40 °C and freeze-dried for 48 hours at a yield of 9 g of dried extracts. The dried extracts were stored at -4 °C until analysis.

4.3.2 Materials (Cell lines), and Maintenance

The human cancer cell lines UACC62, MCF7 and PC3 were acquired from the NCI as part of the framework collaborative research program between CSIR and NCI. HCT116 and WI-38 cell line (normal Human Lung Fibroblast) were obtained from ECACC (European Collection of Authenticated Cell Cultures, UK). Cell lines were routinely maintained as a monolayer cell culture at 37 °C, 5% CO₂, 95% air and 100% relative humidity in RPMI containing 5% foetal

bovine serum, 2 mM L-glutamine and 50 µg/ml gentamicin. Emetine, Parthenolide, glassware, apparatus and other materials used for our experiments were sourced from reputable supplies that include Merck, United Scientific, Minema, B&M Scientific, and Lasec)

4.3.3 Protocol for the Growth Inhibitory Activity Measuring of five *Medicago sativa* L Extracts

The SRB Assay was developed by Philip Skehan and others that were chosen for its rapidity, sensitivity, and low cost of materials (Skehan et al., 1990). For the anti-cancer screening experiment, the cells (3-19 passages) were inoculated in a 96-well microtiter plates at plating densities of 7-10 000 cells/well. The plates were then incubated for 24 hours at 37 ° C with 5% CO₂, then After the 24-hour incubation, the cells were treated with the experimental drugs/extracts which were previously dissolved in DMSO and diluted in medium to produce five concentrations. Cells without drug treatments served as control. The blank contains complete medium without cells. Parthenolide was used as a standard. The plates were incubated for 48 hours after the addition of the compounds. Viable cells were fixed to the bottom of each well with cold 50% trichloroacetic acid, washed, dried, and dyed by SRB. Unbound dye was removed, and protein-bound dye was extracted with 10 mM Tri's base for optical density determination at the wavelength 540 nm using a multi-well spectrophotometer. Data analysis was performed using GraphPad Prism software. 50% of cell growth inhibition (IC₅₀) was determined by non-linear regression.

4.3.4 Protocol for the Cytotoxicity Activity Measuring of five *Medicago sativa* L Extracts

For screening experiment, the cells (21-50 passages) were inoculated in a 96-well microtiter plates at plating densities of 10 000 cells/well and were incubated for 24 hours at 37 ° C with 5% CO₂, then after 24 hours, the cells were treated with the experimental drugs which were previously dissolved in DMSO and diluted in medium to produce five concentrations. Cells without drug treatment served as control. The blank contains complete medium without cells. Emetine was used as a standard. The plates were incubated for 48 hours after addition of the compounds. Viable cells were fixed to the bottom of each well with cold 50% trichloroacetic acid, washed, dried, and dyed by SRB. The unbound dye was removed, and protein-bound dye was extracted with 10 mM Tri's base for optical density determination at the wavelength 540 nm using a multi-well spectrophotometer. Data analysis was performed using GraphPad

Prism software. 50% of cell growth inhibition (IC₅₀) was determined by non-linear regression.

4.4 RESULTS

4.4.1 The in-vitro growth inhibitory effect experimental Results:

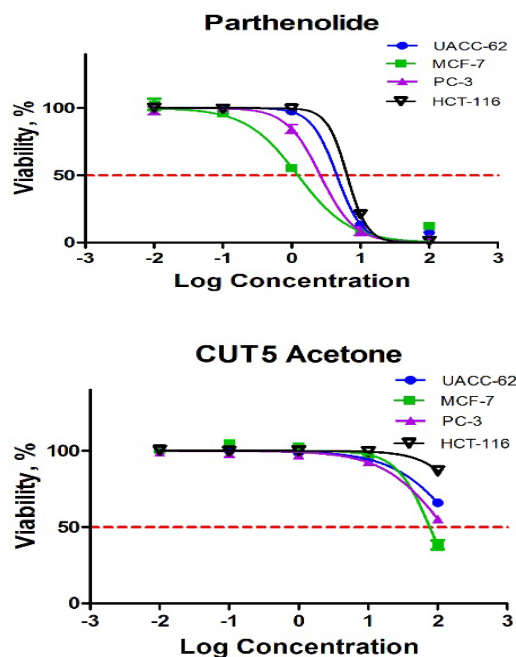


Fig. 4.2 The graph of dose-response curve of five concentrations of Alfalfa Acetone extract against four cancer cell lines. The acetone (100 $\mu\text{g/ml}$) extracts exhibited a weak anti-cancer activity towards the breast cancer cell line/ MCF-7 (3.4 and 3.5) with IC₅₀ of 76.81 $\mu\text{g/mL}$.

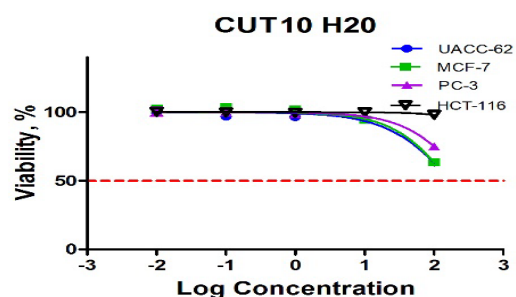


Fig. 4.4 The graph of dose-response curve of five concentrations of Alfalfa water extract against four cancer cell lines. The water extract is therefore inactive according to CSIR's Standard criteria.

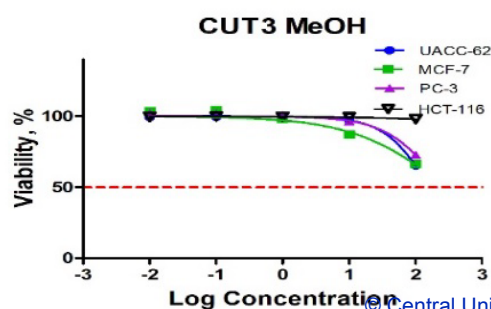


Fig. 4.6 The graph of dose-response curve of five concentrations of Alfalfa methanol extract against four cancer cell lines. The methanol extract is therefore inactive according to CSIR's Standard criteria.

Fig. 4.1 The graph of: Parthenolide's (Control) growth inhibition against five cancer cell lines. The sample were tested at five different concentrations presented as a Log concentration, where: 2.0 = 100 $\mu\text{g/ml}$, 1.0 = 10 $\mu\text{g/ml}$, 0.0 = 1 $\mu\text{g/ml}$, -1.0 = 0.1 $\mu\text{g/ml}$, and -2.0 = 0.01 $\mu\text{g/ml}$. The dose-response curve of the control serves as the standard of measuring the anti-cancer effects of Alfalfa extracts. The IC₅₀ (half maximal inhibitory concentration) is indicated by the red

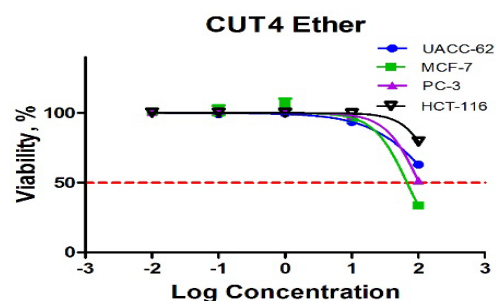


Fig. 4.3 Fig. 4.3 The graph of dose-response curve of five concentrations of Alfalfa Diethyl-ether extract against four cancer cell lines. 100 $\mu\text{g/ml}$ of diethyl-ether extract imposed a weak anti-neoplastic activity towards the breast cancer cell line/ MCF-7 with IC₅₀ of 67.7 $\mu\text{g/mL}$.

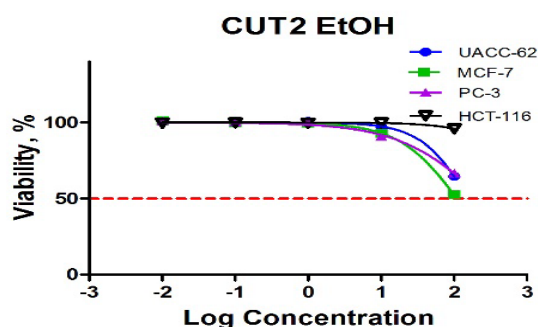


Fig. 4.5 The graph of dose-response curve of five concentrations of Alfalfa ethanol extract against four cancer cell lines. The ethanol extract is therefore inactive according to CSIR's Standard criteria.

4.4.2 The in-vitro Cytotoxicity effect experimental Results:

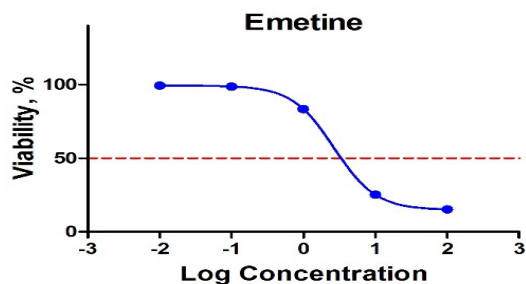


Fig. 4.7 The graph of cytotoxicity measuring of Emetine (control). The CSIR's Standard Cytotoxicity criteria considers samples with IC₅₀ of more than 100 µg/ml a low hazard/low potency, while samples that have IC₅₀ of less than 5 µg/ml are regarded as high hazard / potent. Emetine recorded IC₅₀ of 2.66 µg/ml as thus it exhibited a high cytotoxicity hazard.

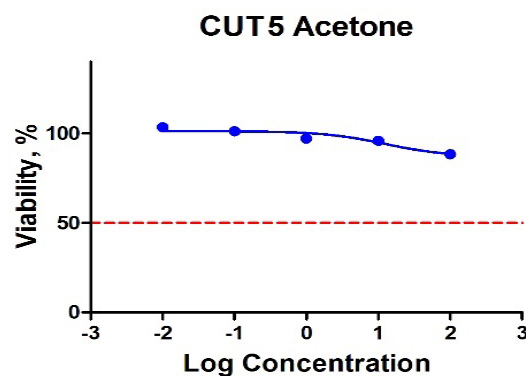


Fig. 4.8 The graph of cytotoxicity measuring of the Acetone extract of Alfalfa. The extract exhibited low hazard cytotoxicity effect.

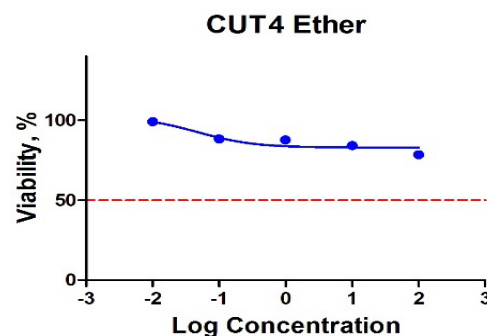


Fig. 4.9 The graph of cytotoxicity measuring of the Diethyl-Ether extract of Alfalfa. The extract exhibited low hazard cytotoxicity effect according to the CSIR's IC₅₀ criteria.

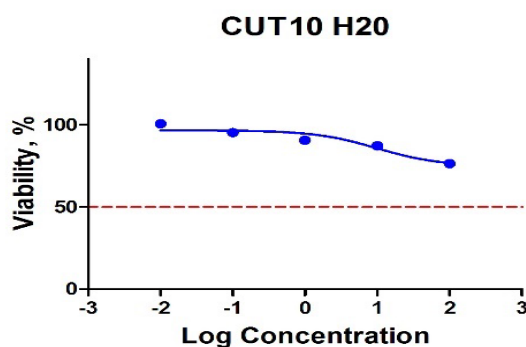


Fig. 4.10 The graph of cytotoxicity measuring of the Diethyl-Ether extract of Alfalfa. The extract's cytotoxicity effect according to the IC₅₀ criteria can be regarded as low hazard.

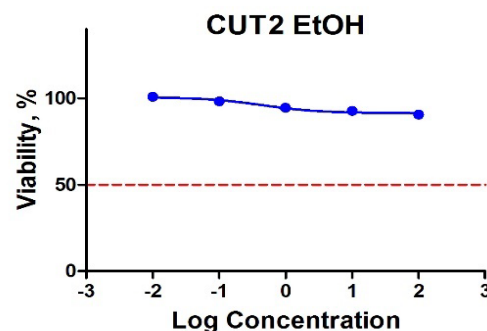


Fig. 4.11 The graph of cytotoxicity measuring of the ethanol extract of Alfalfa. The extract's cytotoxicity effect according to the IC₅₀ criteria can be regarded as low hazard.

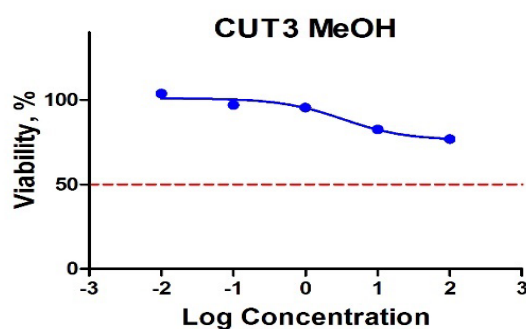


Fig. 4.12 The graph of cytotoxicity measuring of the methanol extract of Alfalfa. The extract exhibited low hazard cytotoxicity effect according to the CSIR's IC₅₀ criteria.

4.5 DISCUSSION AND CONCLUSION

The plant material (*Medicago sativa L.*) was extracted using five solvents: ethanol (sample name: CUT2 EtOH), methanol (CUT3 MeOH), diethyl-ether (CUT4 ether), acetone (CUT5 acetone), and water (CUT10 H₂O). Following treatments, optical density (OD) was determined at the wavelength of 540 nm using a multi-well spectrophotometer. Data analysis was performed using GraphPad Prism software. 50% of cell growth inhibition (IC₅₀) were determined by non-linear regression. The readings and graphical readings of tests are recorded here as Figures 4.1 to 4.12. According to criterion of the CSIR, the sample is considered weak if parameter IC₅₀ for two or three cell lines is more than 15 µg/mL, but less than 100 µg/mL. Therefore, all tested samples can be said to be inactive (Figures 4.4 to 4.6). The diethyl-ether and acetone extracts displayed some weak activity towards the breast cancer cell line/MCF-7 (Figures 4.2 and 4.3) with IC₅₀ of 67.7 µg/mL and 76.81 µg/mL, respectively. The experimental results correlate with the ethno-pharmacological reports that Alfalfa possess weak chemo-therapeutic potential.

Emetine is a pyridoisoquinoline that is used clinically as an emetic, antiprotozoal, antiviral, antimalarial, and antineoplastic agent. Emetine is a natural alkaloid that extracted from the European Ivy (*Hedera helix*), and *Alagium longiflorum*. The cytotoxicity effects of *Medicago sativa L.* and Emetine were tested by the Sulforhodamine B (SRB) assay using the WI38 cell line (normal Human Foetal Lung Fibroblast from the ECACC). The CSIR's Standard Cytotoxicity criteria considers samples with IC₅₀ of more than 100 µg/ml a low hazard/low potency, while samples that have IC₅₀ of less than 5 µg/ml are regarded as high hazard/potent. The cytotoxicity curves (Figures 4.7 to 4.12) is the recording of the cytotoxicity potency of our standard: Emetine, and the experimental results confirms that all the tested extracts of alfalfa exhibited weak-hazard cytotoxicity (Figures 4.7 to 4.12).

Despite modern advances in clinical care, the cost of treatments has been reported by the AICR to be costing consumers a staggering US\$895 billion per year. The cost of treating cancer is not only from the hospitals/clinics (diagnosis, drugs, treatment, and admission fees) but also there are non-healthcare costs related to being a cancer patient. There is enough evidence that many clinical cancers treatments cause for example alopecia and many other physio-psychological side effects that include: weight loss, pain, and fatigue.

There are also other costs related to cancer care and management, for example, the costs of attending treatment sessions. In the year 2003, Rick Simpson was diagnosed with a form of

skin cancer called basal-cell carcinoma. Acting from hearsay evidence that *Cannabis sativa* is a potent therapeutic against cancer, Rick decided to apply the oil he extracted from *Cannabis sativa* on the affected area and covering it with a bandage for several days, following the application of the oil, Rick removed the bandage to find the cancer gone and the area was healed. Mr Simpson claims these actions were motivated by the scientific validation study published by the National Cancer Institute (NCI), whereby THC (delta-9 tetrahydrocannabinol) was demonstrated to kill cancer in mice. The experimental screening of medicinal plants for therapeutic is central to drug development research because it avails empirical data that scientifically validate ethnobotanical reports of the therapeutic potency of some plants of medicinal use.

Medicago sativa L is a famous plant that grows easily anywhere around the world, with common names that includes Alfalfa, Lucerne, Buffalo Herb, Purple Medic/Medick/Medicule and many others (Krakowska et al., 2017). Alfalfa is mostly used in agriculture as a fodder crop, where it provides beneficial nourishment to animals and humans. The Purple Medic plant also has environmental benefits as well; it has been used to prevent soil erosion for example by binding the soil particles together. Although the plant is safe to be consumed by animals and humans, in literature it is reported that the seeds contain L-canavanine, which is an amino acid that is detrimental to patients with the autoimmune disease. Otherwise *Medicago sativa* has been regarded to be beneficial for health and wellness, where many experimental and ethnobotanical reports demonstrated therapeutic effects towards diabetes (lowering hyperglycaemia), lowering cholesterol, acting as a diuretic, and to increase lactation and to promote menstruation. Further testing of the chemo-therapeutic potency of alfalfa is recommended following the identification of active compounds.

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CHAPTER V- EXPERIMENT IV:
ANTI-MICROBIAL TESTING TOWARDS FIVE CLINICAL
PATHOGENS

5.1 ABSTRACT

Makhele Thapelo Simon, Makhoahle Pakiso Moses, and Mashele Sitheni Samson

The *In-Vitro* bioactivity testing of five *Medicago sativa* L. (alfalfa/Lucerne) leaf extracts for Anti-Microbial potential, and cytotoxicity screening against African Green Monkey Kidney cells (Vero Cells)

Anti-Microbial Drug Resistance (AMR) in pathogenic microbial organisms produced a perpetual threat to global public health with 1.27 million deaths in 2019 alone, but it also stimulated extensive searching of microbicidal and germicidal agents that are cheap and easily accessible that also produces comparatively fewer side effects to clinical drugs. The newly isolated and tested phenyl-propanol derivative by Ngah et al, *irisdichototin G* from *Tabernaemontana incospicua* Stapf. (*Apocynaceae*), not only displayed significant antimicrobial effects against the infectious *Haemophilus influenzae* 9435337A with the Minimum Inhibitory Concentration (MIC) of 62.5 µg/mL, but also provides potential proof-of-ability towards combating the AMR pandemic using medicinal plants. The *in-vitro* anti-microbial activity testing of five Alfalfa leaf extracts was evaluated using the Broth Micro-Dilution Assay and the colorimetric Microplate Alamar Blue Assay (MABA). The clinical antimicrobials Gentamicin, Vancomycin and Fluconazole were used as controls. Cytotoxicity assays are employed to determine whether cells continue to proliferate after exposure to a test compound for a specific time period. The 3,4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to assess Vero cell viability following exposure to five *Medicago sativa* L. extracts. The tested extracts exhibited anti-microbial activity against the facultative anaerobe and gram-positive *Streptococcus pneumoniae* and the commensal inhabitant of our gastrointestinal tracts *Enterococcus faecalis* with the MIC of < 0.125 mg/mL and < 0.15 mg/mL, respectively (Table 5.1). Vero cells treated with hexane, diethyl ether, and water extracts induced no decrease in the total number of cells compared to the untreated control. However, an increase of total number of dead cells were observed after methanol, and butanol extracts treatment at 200 µg/mL. The global impact of AMR is wide and adverse, causing extended hospitalizations that amount to higher medical bills and high mortality rates. Therefore, the clinical need for novel antimicrobials is urgent and edicts more testing of compounds, analogues, and derivates from medicinal plants to meet the needs of the UN's SDG number 3.

Keywords: Anti-Microbial Drug Resistance (AMR). Minimum Inhibitory Concentration (MIC). Microplate Alamar Blue Assay (MABA). 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT).

5.2 BACKGROUND

The pathophysiologic basis concerning the aetiology of diseases, has since marvelled ancient healers and scientists' centuries before the times of Lister, Koch, and Pasteur. But most recently, many people have started to discuss not only the effectiveness, but also the safety (side-effects, toxicity, and resistance) of synthetic drug-based disease preventing and disease-treating approaches of our modern medicine. Coronavirus Disease 2019 (COVID-19) is a viral infection that caused a severe acute respiratory syndrome, hyper-inflammatory response, vascular damage, angiogenesis, thrombosis and microangiopathy that dictated the national lockdowns in the year 2020 (Stasi et al., 2020). Anti-Microbial Drug Resistance (AMR) describes the condition where the maximal level of the antibiotic drug/agent that can be tolerated by the host, does not halt, or slow down the growth and development of the infecting organism(s) (Whalen, 2009:354-356). The imposing effect of anti-microbial drug resistance is observed in regions such as in South Asia, whereby every five minutes a newborn child dies from sepsis that is caused by an antibiotic-resistant pathogen (Bhutta, 2008). The innovations of Alexander Fleming and others are no longer enough a deterrent against infectious agents and the diseases that they cause. The pathogen's ability to evolve and adapt has rendered the life-saving magic of many anti-microbial agents as ineffective and hazardous to the health and wellness of the sick. The increasing number of AMR genotypes and phenotypes is an evolutionary response to the widespread use of anti-microbial drugs; the global connectedness of humans from diverse environments also supports the spreading of pathogens with antimicrobial-drug resistance (Michael et al., 2014). Anti-microbial drug resistance can be inherent or acquired (Whalen, 2009:354-356).

Despite apparent advances in healthcare systems and healthcare technologies, nosocomial infections remain a global threat to healthcare across the globe (Ashraf et al., 2020). Common prevalent nosocomial infections include intravenous line-associated blood stream infections, catheter-associated urinary tract infections, surgical site infections and ventilator-associated pneumonia (Khan et al., 2017). Nosocomial pneumonia, vascular access-related bacteraemia and surgical wound infections have caused the most illness and deaths in hospitalized patients. And as thus making the Intensive Care Units (ICU) the axis of antibiotic resistance

(Weinstein, 1998). The pharmaceutical research and development failed to meet the clinical needs of new and effective anti-microbial agents (Boucher et al., 2013; Kinch et al., 2014). The existing literature imposes an urgent need for new and alternative anti-microbial drugs against pathogenic micro-organisms (Luther et al., 2019). While superbugs (Antimicrobial Drug-Resistant Pathogens) kill 700 000 people a year (World Health Organization, 2020), the insect world also hold a potential bioactivity against biofilm as in the use of the degrading-enzymes of the rat-tailed maggot of the drone fly *Eristalis tenax* (Hirsch et al., 2020). The production of harmonine by the harlequin ladybird (*Harmonia axyridis*) is another example of potential antimicrobial agent against malaria and tuberculosis as demonstrated by Christian Rohrich and others (Rohrich et al., 2012).

The most common bacterial Urinary Tract Infection (UTI) in women is caused by *Escherichia coli* (Das, 2020). The infection can occur anywhere in the urinary tract but frequently affects the bladder, urethra, or the kidneys (Das, 2020). Antibiotic drugs are invaluable in the management and treatment of infections but also in the decreasing of deaths associated with infections (WHO, 2020a; WHO, 2020b). At the same time, *Enterococcus faecalis* is responsible for several nosocomial infections of the bloodstream, urinary tract, and of surgical sites (Zaheer et al., 2020). Bacterial Meningitis (caused by *Streptococcus pneumoniae*) is a life-threatening condition that can lead to brain damage with sensory and cognitive impairment (Zhao et al., 2020). The species of *Candida* are notorious in the bloodstreams of hospitalized patients, as well as generally infecting our mouths, skins, and the vagina (Erum et al., 2020). *S. epidermidis* is also well known as a frequent cause of prosthetic joint infections, prosthetic valve endocarditis and neonatal sepsis (Hellmark et al., 2013). The need for effective and cost-friendly therapeutics is urgent and requires a multi-pronged approach that includes looking into our past for clues and solutions (Gil-Gil 2019). The use of plants and plant-derived agents for medicinal purposes by humans is as old as the consumption of plants for food. For humans and animals, plants play a central role in their health and wellness, vitality, and survival. Plants continue to supply a large share of compounds and molecules to the pharmaceutical and food industries.

The gram-negative organisms are notoriously implicated in mostly fatal intra-abdominal infections, urinary tract infections, pneumonia, and bacteraemia. The strong outer-membrane of gram-negative pathogens is responsible for the much globally observed antibiotic-resistance (Luther et al., 2019). The innovation and development by The Zurich University and Polyphor; the so-called Outer Membrane Protein Targeting Antibiotics (OMPTA), is a

novel class of chimeric, cationic, and peptidomimetic antibiotics that have since demonstrated therapeutic activity against antibiotic-resistant gram-negative pathogens (Luther et al., 2019). The anti-microbial resistance pandemic is a global concern affecting both developed and developing countries. There is no alternative but to control the use of antimicrobial drugs, to interpret the mechanisms of resistance and as thus to engineer novel therapeutics against drug-resistant pathogens (Ashraf et al., 2020). The need for effective and cost-friendly therapeutics is urgent and requires a multi-pronged approach that includes looking back into our past (*Sankofa*) for clues and solutions (Ashraf et al., 2020). The use of plants and plant-derived agents for medicinal purposes by humans and animals is old, diverse, and advanced. For humans and animals, plants play a central role in their health and wellness, vitality, and survival as plants continue to supply a large share of beneficial compounds and molecules to the food and the Agri-pharmaceutical industries.

Anti-biotic susceptibility testing is an effective tool in the treatment and management of infections; it allows clinicians to select the appropriate anti-microbial agent and to prescribe with precise accuracy, the bactericidal/bacteriostatic dosage (Baronikova et al., 2004). The prescribed anti-biotic needs to be effective without causing significant or severe negative side effects to the person or animal or plant. The application of anti-microbial susceptibility testing for the evaluation of potential bioactivity of natural products is one of the effective interventions instigated to curb the current surge of global AMR (Schumacher et al., 2018). Cytotoxicity assays are tools used to determine whether cells continue to proliferate after exposure to a test compound for a specific time period. Cytotoxicity is usually assessed by methods that compare either the cellular (enzymatic) function and/or cell integrity of control cells to those exposed to a test compound (Cummings and Schnellmann, 2004). We used a quantitative colorimetric assay based on the metabolic activity of cells to evaluate cell viability after exposure to Alfalfa extracts (MTT assay).

The use of *alfac-facah* for medicinal purposes include neuroprotective, anti-microbial, anti-cancer and cholesterol lowering physiologic activities (Liu et al., 2019). Newly isolated triterpenoid saponins from *M. sativa* L. demonstrated neuroprotective potential against hydrogen peroxide induced SH-SY5Y (Human Neuroblastoma) cell death compared to hydrogen peroxide treated group (Liu et al., 2019). The toluene and methyl tert-butyl ether (MTBE) extracts of *M. sativa* L. demonstrated in-vitro cytotoxic effects on two murine leukaemia cell lines P388, where the extracts also induced programmed cell death by activation of the caspase-3, leading to poly ADP ribose polymerase cleavage (PARP)

(Rafinska et al., 2017). The aim of this chapter was to evaluate the anti-microbial activity and to determine the MIC of Alfalfa (*Medicago sativa L.*) leaf extracts against a panel of five clinical pathogenic strains (*E. coli*, *E. faecalis*, *S. epidermidis*, *S. pneumoniae* and *C. albicans*), using the Broth Micro-dilution method and Microplate Alamar Blue assay.

5.3 MATERIALS AND METHODS

5.3.1 The Test (Plant Material), The Extraction and The Preparation for *in-vitro* Bio-Analysis

The plant materials (Figure 5.1) were collected from a nursery and medicinal plant farm North of Pretoria (South Africa) with the Batch Number MH 71(10 kg). The plant material was cultivated by Zizameleni Farming based in Mamogalieskraal, Northwest Province of South



Fig 5.1:
Image of
Medicago Sativa L.
(Alfalfa or

Africa. The test material was cultivated using regenerative natural farming principles and the fertilizers used were all natural and certified organics. The material was air-dried and stored in a cool dry area away from light and heat. The dry, grassy, and pale green leaves were extracted using five solvents: butanol, diethyl-ether, hexane, methanol, and water. Sixty (60) grams of powdered plant material were extracted in 1 litre (1 000 mL) of every solvent. The extracts were filtered using the Buchner funnel and Whatman no.1 filter paper. The extracts were frozen at -40 °C and freeze-dried for 48 hours at a yield of 9 g of dried extracts. The dried extracts were stored at -4 °C until analysis.

5.3.2 The *in-vitro* bioactivity testing of five Alfalfa leaf extracts against pathogenic micro-organisms.

There are countless approaches and techniques employed for the screening of biological extracts for therapeutic potential use as antimicrobial agents. The Broth Micro-dilution susceptibility method in a 96-well microtiter plate, has become the preferred method for drug susceptibility testing due to its small sample requirements and high-throughput rate (Pauli et al., 2005). Microplate Alamar Blue assay (MABA) is a colorimetric assay that can be utilised for drug-susceptibility testing based on oxidation/reduction reactions (Collins and Franzblau, 2005). This assay uses an oxidation/reduction indicator; Alamar Blue (CellTiter-Blue®), that changes colour from blue to pink, indicating bacterial growth. The colour change can be read

visually and quantified fluorometrically by excitation at 530 nm and detecting emission at 590 nm (Collins and Franzblau, 2005).

The Broth Micro-Dilution Assay was used to evaluate the anti-microbial activity of *Medicago sativa* L. leaves against five clinical strains (*E. coli*, *E. faecalis*, *S. epidermidis*, *S. pneumoniae* and *C. albicans*). The method is favoured for its highly accurate results, availability of testing plates and standard reagents and the possibility of reading results both qualitatively (colour changes) and quantitatively by Spectrophotometric techniques (Balouri et al., 2015). The twofold dilutions of the candidate anti-microbial agent (plant leave extracts) were incubated in broth with the 0.5 McFarland standard adjusted organism suspension. The anti-microbial activity is confirmed against the clinical antimicrobial agent (Vancomycin/Gentamicin). Following the 24-hour incubation period, 20 µL of CellTiter-Blue® Reagent (Promega) were added to each well and incubated for 1 hour. Wells were observed for colour change, where a blue colour represented no growth (non-viable cells) and a pink colour represented growth (viable cells). The fluorescence was read at excitation and emission wavelengths of 535 and 590 nm, respectively, using a BioTek® SYNERGY Mx fluorometer (Winooski, VT, USA).

Fluorescence readings of 96-well plates were measured at excitation and emission wavelengths of 535 and 590 nm after addition of CellTiter blue. Percentage inhibition for the extracts was calculated for some extracts showing inhibition of growth; however not for those that produced slight pink colouration.

Calculation used to determine percentage inhibition:

$$(1 - (\text{Fluorescence at 535/590 nm of test extract (fluorescence corrected by subtracting the fluorescence produced by the extract)} / \text{Fluorescence at 535/590 nm microbe growth control})) \times 100$$

5.3.3 Materials for the anti-microbial bio-activity testing of Alfalfa extracts

Enterococcus faecalis, *Staphylococcus epidermidis*, and *Escherichia coli* (clinical strains) were grown in Mueller-Hinton (MH) broth (Merck, USA). *Candida albicans* was maintained in Malt Extract broth (Merck, USA). *S. pneumoniae* was grown in Brain-Heart Infusion broth (Merck, USA). One microorganism colony, from an overnight streak plate, was inoculated in broth (10 mL) and allowed to grow for 16 hours (log growth phase) at 37 °C. Control drugs used: Gentamicin sulphate and Vancomycin hydrochloride (Sigma, USA) used as positive

controls against gram-negative and gram-positive bacteria, respectively. Fluconazole (Sigma, USA) was used as positive controls against *C. albicans*. Antibiotics were dissolved in ddH₂O at stock concentrations of 2 mg/mL and filter sterilized (0.2 µM filter). Working concentrations were prepared in broth, depending on the antibiotics' MIC values. Test Extracts were dissolved in Dimethyl Sulfoxide (DMSO) at stock concentrations of 100 mg/mL. Working concentrations of 4 mg/mL were prepared in MH broth/Malt Extract/Brain-Heart infusion broth.

5.3.4 The Anti-microbial bio-activity testing Procedure (Broth Micro-Dilution Assay)

50 µL of MH broth/Malt Extract/Brain-Heart infusion broth were added to all test wells (i.e. plant extracts and antibiotics), except for the highest plant extract and antibiotic concentration wells to which 100 µL of the working concentrations were added. Serial dilutions were prepared for the plant extracts: 2 mg/mL to 125 µg/mL and for antibiotics: 64 to 0.25 µg/mL. The cultures were assessed and adjusted to a 0.5 McFarland standard (absorbance at 600 nm = 0.08-0.1; equivalent to $\sim 1.5 \times 10^8$ cells/mL) and 50 µL added to each test well.

5.3.5 Preparation of Controls

Antibiotic/medium control (50 µL broth + 50 µL of highest antibiotic concentration). Extract colour control (50 µL broth + 50 µL of highest plant extract concentration). 4% DMSO control (50 µL broth + 50 µL 8% DMSO). Microorganism control (50 µL broth + 50 µL microorganism). Plates were sealed with microplate sealing tape and incubated at 37°C for 24 hours.

5.3.6 CellTiter-Blue® assay

After treatment, 20 µL of CellTiter-Blue® Reagent (Promega) were added to each well and incubated for one hour. Wells were observed for colour change, where a blue colour represented no growth (non-viable cells) and a pink colour represented growth (viable cells). The fluorescence was read at excitation and emission wavelengths of 535 and 590 nm, respectively, using a BioTek® SYNERGY Mx fluorometer (Winooski, VT, USA).

5.3.7 The *in-vitro* cell viability testing of five Alfalfa leaf extracts against Vero Cells

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is commonly used to assess cell viability. The MTT assay is a quantitative colorimetric assay based on the metabolic activity of cells. The yellow water-soluble tetrazolium dye is reduced to a dark

blue-to-purple insoluble formazan product by mitochondrial enzymes of viable cells. The insoluble formazan product is dissolved in dimethyl sulfoxide (DMSO) and can be measured spectrophotometrically at 540 nm. The amount of product produced is proportional to the number of viable cells (Lema et al., 2011; Vega-Avila and Pugsley., 2011). The limitation of this method is that other factors can influence the metabolic activity of cells and therefore we cannot accurately measure cell death (Lema et al., 2011).

Measuring the integrity of cell membranes is an alternative reliable method that can be used to assess cell viability (Lema et al., 2011) Dual staining with Hoechst and Propidium iodide (PI) was used to assess viable and nonviable cells, respectively. Hoechst 33342 is a membrane permeant dye that binds to the minor grooves of double-stranded DNA. Hoechst 33342 is excited at a wavelength of 350 nm and emits a blue fluorescence at 461 nm. Because Hoechst is a cell permeant dye it stains all nucleic acids and therefore give a total cell count, i.e. live plus dead cells (Chazotte, 2011). PI is red fluorescent molecule that binds to DNA and is excited at 536 nm and emits light at 623 nm. PI uptake is dependent on the integrity of the cell membrane and cannot enter cells with intact membranes. The uptake and exclusion of this dye can therefore be used to discriminate between viable and nonviable cells (Ciancio et al., 1988; Crowley et al., 2016).

5.3.8 Materials and Methods

Vero cells were purchased from Cellonex, South Africa. DMEM low glucose cell culture medium and FBS were purchased from GE Healthcare Life Sciences (Logan, UT, USA). PBS with and without Ca^{2+} and Mg^{2+} and trypsin-EDTA was purchased from Lonza (Walkersville, MD, USA). Bis-benzamide H 33342 trihydrochloride (Hoechst) and propidium iodide (PI) were purchased from Sigma (St. Louis, MO, USA).

5.3.9 Image acquisition and analysis

Images were acquired using the ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices). Plate acquisition setup for a 96 well plate at a magnification of 10 times was used. Spatial distribution for acquisition was set at 9 sites per well (3 by 3). The 9 images per site covers roughly 70% of the total well area. Filters used included DAPI (for Hoechst) and Texas Red (for PI). Analysis of acquired images was performed using the Multi Wavelength Cell Scoring analysis module of the MetaXpress version 6.1 High-Content Image Acquisition and Analysis Software.

Plate 1	1	2	3	4	5	6	7	8	9	10	11	12
A	Control	Control	Hexane				Diethyl ether					
B	Control	Control										
C	Control	Control										
D	Control	Control										
E	Control	Control	Methanol				Butanol					
F	Control	Control										
G	Control	Control										
H	Control	Control										
	Note: only applicable for C. albicans											
Plate 2	1	2	3	4	5	6	7	8	9	10	11	12
A	Control	Control	Water				[2] rep 1	[1] rep 1	[0,5] rep 1	[0,25] rep 1	[0,125] rep 1	
B	Control	Control					[2] rep 1	[1] rep 1	[0,5] rep 1	[0,25] rep 1	[0,125] rep 1	
C	Control	Control					[2] rep 1	[1] rep 1	[0,5] rep 1	[0,25] rep 1	[0,125] rep 1	
D	Control	Control					[2] rep 1	[1] rep 1	[0,5] rep 1	[0,25] rep 1	[0,125] rep 1	
E	AB Control	Medium Control	DMSO control	Growth control	Positive control (Vancomycin/ Gentamicin)							
F												
G												
H												
	Note: only applicable for C. albicans											
Plate 3	1	2	3	4	5	6	7	8	9	10	11	12
A	AB Control	Medium Control	DMSO control	Growth control	Fluconazole							
B												
C												
D												
E	AB Control	Medium Control	DMSO control	Growth control	Fluconazole							
F												
G												
H												

Fig. 5.2: The 96-well Micro-Plate Layouts (Dilutions and Treatments) for Antimicrobial Activity testing of Alfalfa Extracts

5.4 RESULTS

5.4.1 The in-vitro anti-microbial experimental results of five *Medicago sativa L.* extracts against a panel of five clinical pathogens (*E. coli*, *E. faecalis*, *S. epidermidis*, *S. pneumoniae* and *C. albicans*) using the Broth Micro-dilution method and Microplate Alamar Blue assay.

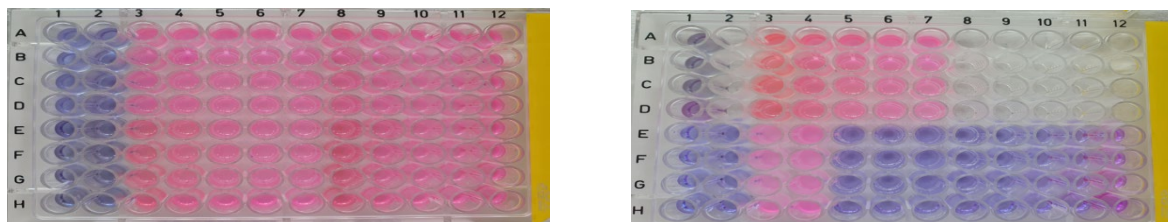


Fig. 5.3: Plate 1 and Plate 2 of Anti-microbial activity testing and MIC determination of Alfalfa extracts against *E. coli*. Alamar Blue (CellTiter-Blue®) changes colour from blue to pink, to indicate bacterial growth. The colour change can be quantified fluorometrically. Plate 1 and Plate 2 shows no microbial inhibition caused by treatment with Alfalfa extracts. The Positive control (Gentamicin) caused a colour change to blue, confirming that bactericidal effects against *E. coli*.

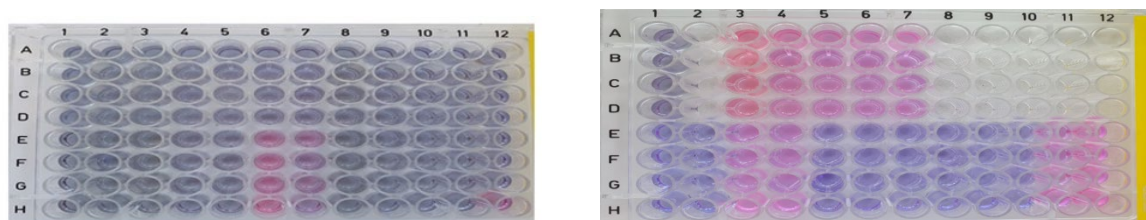


Fig. 5.4: Plate 1 and Plate 2 of Anti-microbial activity testing and MIC determination of Alfalfa extracts against *E. faecalis*. Alamar Blue (CellTiter-Blue®) changes colour from blue to pink, to indicate bacterial growth. The colour change can be quantified fluorometrically. Plate 1 and Plate 2 microbial inhibition caused by treatment with by the Butanol, Diethyl ether and Methanol extracts of Alfalfa with the MIC of <0,125 mg/mL. The Positive control (Vancomycin) caused a colour change to blue, confirming that bactericidal effects against *E. faecalis*.

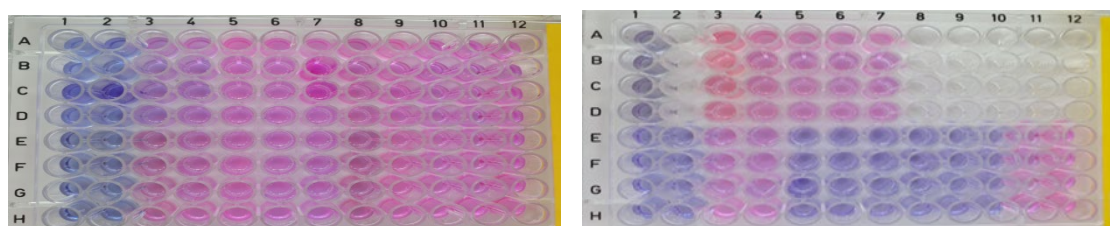


Fig. 5.5: Plate 1 and Plate 2 of Anti-microbial activity testing and MIC determination of Alfalfa extracts against, *S. epidermidis*. The Positive control (Vancomycin) caused a colour change to blue, confirming that bactericidal effects against *S. epidermidis*. All the tested extracts exhibited no microbial inhibition at all tested dosages.

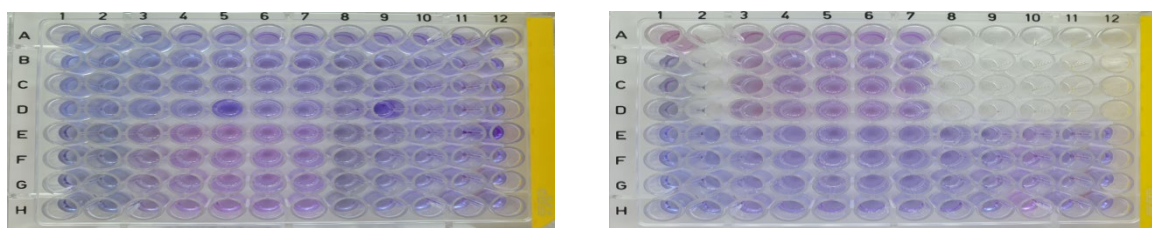


Fig. 5.6: Plate 1 and Plate 2 of Anti-microbial activity testing and MIC determination of Alfalfa extracts against *S. pneumoniae*. Vancomycin and Alfalfa extracts inhibited *S. pneumoniae* growth. This evidence correlates with the ethnobotanical claims that *M. sativa L* exhibits anti-microbial bioactivity against pathogenic organisms with the MIC of <0,5 mg/mL for the methanol extract and <0,125 mg/mL for all the other extracts.

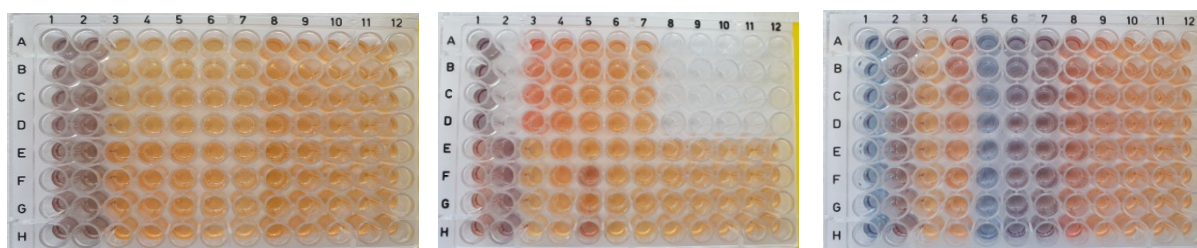


Fig. 5.7: Plate 1, Plate 2 and Plate 3 of Anti-microbial activity testing and MIC determination of Alfalfa extracts against *C. albicans*. Alfalfa extracts inhibited no growth inhibition of *C. albicans*. The positive control Fluconazole exhibited inhibitory effect with the MIC of 0.25 mg/mL.

5.4.2 The in-vitro cytotoxicity Screening of Alfalfa Extracts in Vero Cells

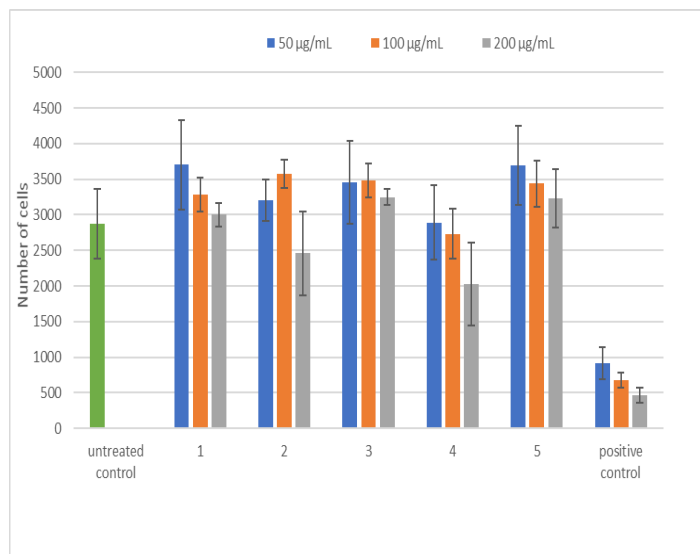


Fig. 5.8 Total number of cells (live + dead) after treatment with extracts and positive control (Melphalan). Plant extracts are plotted as: 1(hexane), 2 (diethyl ether), 3 (methanol), 4 (butanol) and 5 (water). Different colours indicate different concentrations

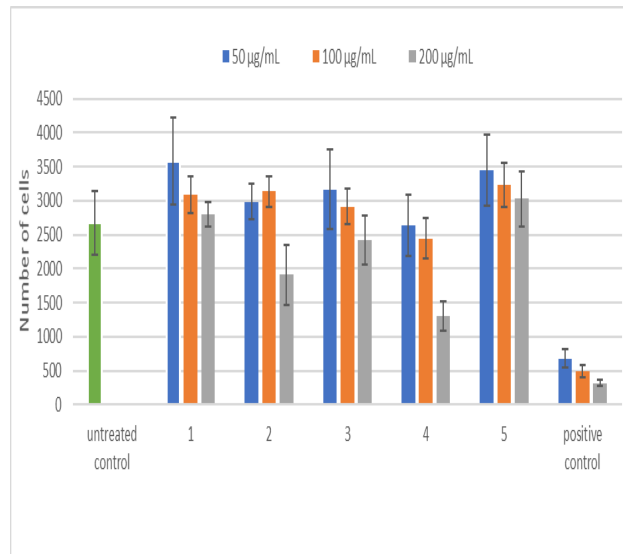


Fig. 5.9 Number of live cells after treatment with extracts and positive control (Melphalan). Plant extracts are plotted as: 1(hexane), 2 (diethyl ether), 3 (methanol), 4 (butanol) and 5 (water). Different colours indicate different concentrations.

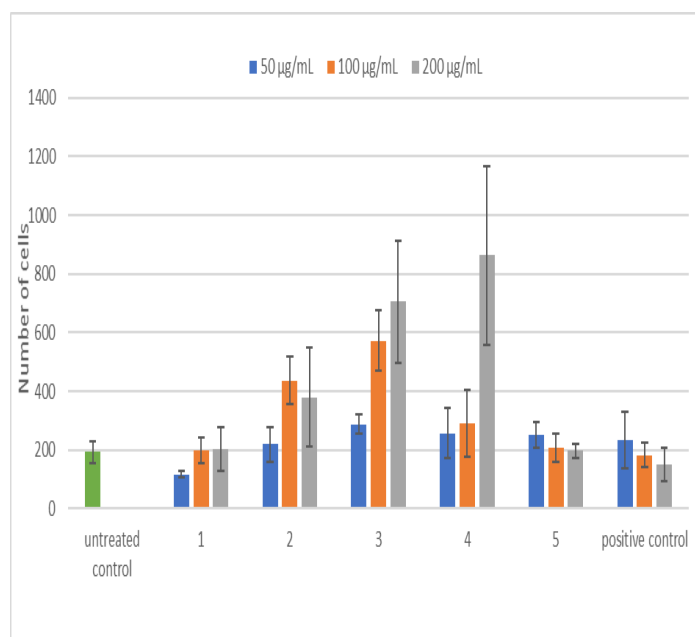


Fig. 5.10 Number of cells with PI positive staining (dead cell number) after treatment with extracts and positive control (Melphalan). Plant extracts are plotted as: 1(hexane), 2 (diethyl ether), 3 (methanol), 4 (butanol) and 5 (water). Different colours indicate different concentrations.

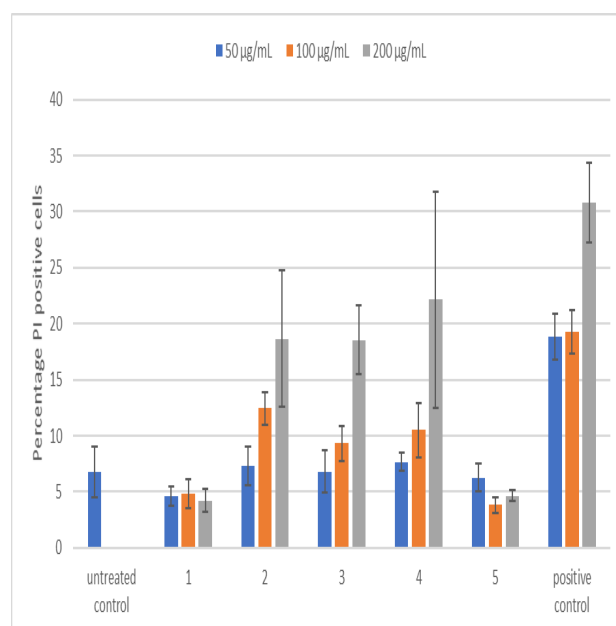


Fig. 5.11 Percentage PI positive after treatment with extracts and positive control (Melphalan). Plant extracts are plotted as: 1(hexane), 2 (diethyl ether), 3 (methanol), 4 (butanol) and 5 (water). Different colours indicate different concentrations.

5.5 DISCUSSION AND CONCLUSION

Antibiotic drugs are invaluable in the management and treatment of infection but also in decreasing deaths associated with infections (WHO 2020). All the *Medicago sativa* L. Leaves Extracts did not show inhibitory activity against *E. coli* (Figure 5.3). *Medicago sativa* L. leave extracts (hexane, diethyl ether, butanol) exhibited growth inhibition against *E. faecalis* with MIC (Minimum Inhibitory Concentration) of < 0.125 mg/mL (Figure 5.4). This experimental result validates the ethnobotanical indication that *Medicago sativa* L. leaves possess the potential anti-microbial activity against pathogenic organisms (Liu et al., 2019). *Medicago sativa* L. Leaves Extracts (hexane, diethyl ether, butanol) demonstrated inhibitory activity against *S. pneumoniae* with MIC (Minimum Inhibitory Concentration) of < 0.125 mg/mL (Figure 5.6).

The experimental finding establishes a scientific fact that plants can inhibit the growth and multiplication of disease-causing micro-organisms. This finding gives the religious habit of drinking herbal tea for health and wellness more value and potentiates the use of *Medicago sativa* L. as a possible alternative non-drug anti-microbial. The species of *Candida* are notorious in the bloodstreams of hospitalized patients, as well generally infecting our mouths, skin, and the vagina (Erum, 2020). *S. epidermidis* is also well known as a frequent cause of prosthetic joint infections, prosthetic valve endocarditis and neonatal sepsis (Hellmark, 2012; WHO, 2020b). All the *Medicago sativa* L. leave extracts did not show any inhibitory activity against *C. albicans* and *s. epidermidis* (Figures 5.7 and 5.5).

Cytotoxicity assays are tools used to determine whether cells continue to proliferate after exposure to a test compound for a specific time. Cytotoxicity results are shown and discussed for a cell density of 5 000 cells/100 μ L/well. The total number of cells (live + dead): Vero cells treated with hexane (1), methanol (3), and water (5) extracts did not show a decrease in the total number of cells compared to the untreated control (Figure 5.8). A decrease in the total number of cells was observed for diethyl ether (2) and butanol (4) extracts at 200 μ g/mL (Figure 5.8).

The number of live cells: Vero cells treated with hexane (1) and water (5) extracts did not show a decrease in the number of live cells compared to the untreated control (Figure 5.9). A decrease in the number of live cells was observed for diethyl ether (2), methanol (3), and butanol (4) at a concentration of 200 μ g/mL compared to the untreated control (Figure 5.9).

The number of PI-positive cells (dead cells): Vero cells treated with hexane (1) and water (5) did not show an increase in the number of PI-positive cells (Figure 5.10). Diethyl ether (2), methanol (3), and butanol (4) showed an increase in the number of PI-positive cells (Figure 5.10).

The Percentage PI positive: Vero cells treated with hexane (1) and water (5) did not show an increase in the percentage of PI-positive cells (6). Diethyl ether (2), methanol (3), and butanol (4) showed a dose-dependent increase in the percentage of PI-positive cells (Figure 5.11). We report therefore report that the hexane and water extracts demonstrated no evidence of toxicity or cytostatic activity, while the diethyl ether, methanol, and butanol extracts demonstrated toxicity at 200 µg/mL, but not lower concentrations.

Plants and other natural sources continue to provide a diverse variety of compounds, analogues, and derivatives that can be used to formulate and develop the much-needed anti-microbial drugs that can combat antibiotic-resistant pathogens (Rafinska et al., 2017). Anti-microbial susceptibility testing of medicinal plants is not only used to confirm the ethnobotanical literature but also to evaluate the plant as a prospective anti-microbial agent against drug-resistant clinical pathogens. The use of *alfac-facah* for medicinal purposes includes use as a neuroprotective, anti-microbial, anti-cancer, and cholesterol-lowering agent (Liu et al., 2019). Newly isolated triterpenoid saponins from *M. sativa L.* demonstrated neuroprotective potential against hydrogen peroxide-induced SH-SY5Y (Human Neuroblastoma) cell death compared to the hydrogen peroxide-treated group (Liu et al., 2019). The toluene and methyl tert-butyl ether (MTBE) extracts of *M. sativa L.* demonstrated in-vitro cytotoxic effects on two murine leukaemia cell lines P388, whence the extracts also induced programmed cell death by activation of the caspase-3, leading to poly ADP ribose polymerase cleavage (PARP) (Rafinska et al., 2017).

Despite the existing knowledge of clinical-drug to herbal-drug interactions, medicinal plants continue to play a major role in disease prevention, infection control and drug development (Siddiqui et al., 2020). The regular and consistent consumption of herbal infusions (tea) was a key action that our ancestors used to flavour water and siphon nutrients and secondary metabolites from plants and herbs, trees and shrubs availed by nature or the creator (*Ramaseli, Rammoloki, Tlatlamacholo*). The common methods of extraction that were advanced by our ancestors included among many other infusions of the plant or plant part (dry or green) using sterilized (boiled) water. The approach to processing roots and tree barks

included decoctions, fomentations for the skin, soaking or irrigating specific body area. The primitive hunter was also proficient in dealing with snake and insect bites. In that regard he/she used poultices or cataplasms of macerated plant material like the live leaf or bark of the Tanzanian *Antidesma venosum* E. Mey. ex Tul. (*Phyllanthaceae*), wrapping it against the skin or an area of much swelling.

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CHAPTER VI- GENERAL DISCUSSION AND CONCLUSION/RECOMMENDATIONS

6.1 Summary and brief discussion of the study

The current study conducted experiments to measure the in-vitro therapeutic bioactivity of five *Medicago Sativa L* leaf extracts. From the global state of public health, it is evident that novel-therapeutic interventions are critically needed to address the perpetuating global burden of diseases. The data measured by the Global Burden of Disease Study suggest that more than half of all premature mortalities (55%) are due to ten leading causes of disease. The UN's SGD number three aims to ensure healthy lives and to promote good well-being for all at all ages. The UN through the WHO supports research and development of vaccines and medicines for communicable and non-communicable diseases that primarily affect developing countries like the SADAC countries. Diabetes and hyperglycaemia account for millions of deaths annually, and the numbers are calculated to increase. The weak point of the existing medical care is the inability of current therapeutics to meet the clinical needs of curbing the existing global burden of diseases, and access to affordable medicines that induce less side effects.

The current study's objective was to determine the glucose-utilization activity of five *Medicago sativa L* (Alfalfa) leaf extracts to measure the DPPH scavenging action and anti-inflammatory screening of five alfalfa leaf extracts to evaluate the in-vitro neoplastic cell growth inhibition and cytotoxicity bioactivity of Alfalfa and to establish the anti-microbial bioactivity of five leaf extracts against a panel of five pathogenic microorganisms and the cytotoxicity screening against African Green Monkey Kidney cells (Vero cells). The current study established that consuming alfalfa tea can have therapeutic benefits in low-income countries where access to healthcare is rare for the majority. The glucose-utilization experiments reported in Chapter II, the diethyl-ether and water extracts revealed significant increase in glucose utilisation towards the hepatic HEPG2/C3A cell line (Figures 2.2 and 2.3). The butanol and diethyl-ether extracts produced a significant increase in glucose utilisation in the L6 mouse skeletal muscles cell line (Figures 2.6 and 2.7). The results of this experiment were published in a peer-reviewed journal in 2020 (Appendix A: Manuscript Alpha). The results of the current chapter contribute therapeutic confirmation that is measurable and reliable.

Anti-microbial drug resistance (AMR) in pathogenic micro-organisms is threatening the efficacy of existing therapeutic agents. The low number of approved therapeutics against drug resistant and gram-negative pathogenic micro-organisms indicates the need to increase

the screening of medicinal plants for therapeutic evaluation. Medicinal plants that exhibit potent in-vitro and in-vivo bioactivities can then be explored to extract compounds of medicinal value. The *in-vitro* anti-microbial activity testing of five Alfalfa leaf extracts was evaluated using the Broth Micro-Dilution Assay and the colorimetric Microplate Alamar Blue Assay (MABA). The clinical antimicrobials Gentamicin, Vancomycin and Fluconazole were used as positive controls. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to assess Vero-cell viability following exposure to five *Medicago sativa* L. extracts. The tested extracts exhibited anti-microbial activity against the facultative anaerobe and gram-positive *Streptococcus pneumoniae* and the commensal inhabitant of our gastrointestinal tracts *Enterococcus faecalis* with the MIC of < 0.125 mg/mL and < 0.15 mg/mL, respectively. The results correlate with the ethnobotanical claims that Lucerne can potentially be used as a therapeutic agent against infections.

The existing literature suggests that continued oxidative stress can lead to low grade chronic inflammation and ultimately promote/mediate chronic diseases including cancer, diabetes, cardiovascular, neurological, and pulmonary diseases. The current study measured the therapeutic potential of Alfalfa as an anti-neoplastic and cytotoxic agent (Chapter III), DPPH scavenging and anti-inflammatory screening of leaf extracts. The anti-cancer activity screening of medicinal plants is the first step in discovering natural chemotherapeutics. The diethyl-ether and acetone extracts displayed some weak activity towards the breast cancer cell line/ MCF-7 (3.4 and 3.5) with IC₅₀ of 67.7 µg/mL and 76.81 µg/mL, respectively. All tested extracts of alfalfa exhibited weak-hazard cytotoxicity. The experimental results correlate with the ethno-pharmacological reports that Alfalfa possess some chemo-therapeutic potential. For example, the novel isolated triterpenoid saponins from *M. sativa* L exhibited neuroprotective potential against hydrogen peroxide induced SH-SY5Y (human neuroblastoma cell line) cell death compared to hydrogen peroxide treated group (Liu *et al* 2019). The toluene and methyl tert-butyl ether (MTBE) extracts of *M. sativa* L. demonstrated in-vitro cytotoxic effects on two murine leukaemia cell lines P388, where the extracts also induced programmed cell death by activation of the caspase-3, leading to poly ADP ribose polymerase cleavage (PARP) (Rafinska et al., 2017).

6.2 Conclusions and Recommendations

This study was conducted with the aim of evaluating and reporting the therapeutic bioactivity of *Medicago Sativa L* leaf extracts. The UN's SDG number three seeks to improve public

health to decrease mortality from the top ten leading diseases by the year 2030. The need for easily accessible and cheap therapeutics rationalizes the screening of medicinal plants for the development of the much-needed therapeutics. The experimental results determined by the experiments performed suggest that Alfalfa contains therapeutic compounds that can be explored further. The microbial growth inhibitory effects imposed by *M. sativa*'s extracts, coupled with the significant glucose-utilization activity they exhibited, motivates further experimental evaluation of therapeutic bioactivity testing using *in-vivo* animal models. The therapeutic bioactivity imposed by Alfalfa extracts towards neoplasms, oxidative stress and inflammation validates the habitual consumption of herbal tea for health and wellness. The results of the experiment corroborate and correlates with the existing literature on the therapeutic potential of *Medicago sativa* L, and this study contributes data that have never been determined and reported.

***In-vitro* glucose-utilization activity testing of *Medicago sativa* L. (alfalfa) leaf extracts in L6 and HEPG2/C3A cells**

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ABSTRACT

Diabetes mellitus (DM) is a metabolic condition defined by chronic and abnormally high blood sugar levels (hyperglycaemia). Globally 537 million adults were affected in 2021 with the possibility of further increase in the future. Current treatment is reported to be inefficient in the long run and has caused undesirable side effects which affect those suffering from it. *In-vitro* glucose-utilization activity of alfalfa leaf extracts was done with the aim of confirming the existing circumstantial evidence of the beneficial anti-diabetic effects that are credited to the consistent drinking of alfalfa extract in the form of herbal tea. The glucose utilization activity testing of *Medicago sativa* L. leaf extracts was performed in L6 murine skeletal muscle and the C3A/HEPG2 liver cells. The dry, grassy, and pale green leaves were extracted using five solvents: butanol, diethyl-ether, hexane, methanol and water. The resulting activity was compared with the activity of clinical therapeutics: insulin and metformin. The cytotoxicity of the test material was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT Assay) in L6 and the C3A/HEPG2 liver cells. The results showed that the butanol and diethyl-ether extracts produced significant increase in glucose utilisation towards the L6 cells, while the diethyl-ether and water extracts revealed sizeable increase in glucose utilisation towards the C3A cells. The potency of glucose use imposed by our extracts at all tested ranges did not induce glucose utilization activity greater than

the activity induced by insulin and metformin. The results demonstrated no evidence of significant cytotoxicity toward differentiated L6 and C3A cells. Our experiments support the drinking of alfalfa herbal tea for the management of hyperglycaemia with caution because of the increased glucose-utilization activity induced by *Medicago Sativa* L. extracts. The results of this study warrant further examination of the mechanism of action of alfalfa tea on hyperglycaemia and also identification of active compounds for future development of drug or nutritional supplements towards diabetic care.

KEYWORDS: diabetes mellitus (DM), glucose transporter type 4 (GLUT-4), alfalfa (*Medicago sativa* L.), world health organization (WHO), international diabetes federation (IDF).

1. INTRODUCTION

The polypeptide hormone insulin remains the corner stone of bodily blood glucose-level regulation through the varied mechanisms and actions that can include: the promotion of nitric oxide and endothelin production in endothelial cells; glycogenesis in the liver, in the skeletal muscles, and adipocytes; increased glucose uptake through the glucose transporter type 4 (GLUT-4) receptor in the skeletal myocytes and adipocytes; and also, the downregulation of liver gluconeogenic enzymes [1]. The physiologic state of hyperglycaemia will persist as long as the effects of insulin are disabled. This resultant prolonged state of high blood sugar

levels is diagnosed as diabetes mellitus, a chronic and systematic condition that negatively affects the quality of life of the ones affected and effected [2].

The global incidence of diabetes increased sharply from 108 million to 422 million people with DM in the year 2014 [3]. The World Health Organization (WHO) also confirms that diabetes is accused of causing 1.6 million deaths in the year 2016 alone, while high blood glucose level (hyperglycaemia) is said to have caused 2.2 million deaths in the year 2012. The global incidence of diabetes was figured by the International Diabetes Federation (IDF) to be affecting 537 million adults in the year 2021, a figure that is seen to be increasing steadily [4].

The prevalent undesired outcome of diabetic complications includes limb amputations, blindness, or cataracts (retinopathy), heart attacks (vasculopathy), and kidney failure (nephropathy) [4]. The adverse action of advanced glycation end products (AGE's) such as carboxymethyl lysine and pentosidine is also considered to be a major contributor in diabetic complications [5]. There are four mechanisms by which hyperglycaemia has been shown to cause diabetic complications: activation of the polyol pathway; increased formation of advanced glycosylation end products; activation of Protein Kinase C and the hexosamine pathways [6]. The existing literature demonstrates that diabetic complications may arise from reductive and oxidative stress, as a result of excessive and persistent production of ROS [2].

Adipocytokines in obese individuals are also implicated as the cause of oxidative stress and inflammation, where they can even promote insulin resistance and thus induce Type-2 diabetes (non-insulin dependent diabetes) and cardiovascular diseases [7]. Insulin resistance (IR) refers to a metabolic state where the body's ability to utilize glucose is impaired due to a decreased or abnormal response to insulin and also decreased insulin production [8]. The risk factors for IR include obesity; physical inactivity; a diet that is high in carbohydrates; family history, and ethnicity (as it was found to be more prevalent in Africans, Asian-Americans, Hispanics, American Indians, and so on.).

Septic shock is a life threatening syndrome that follows untreated sepsis, characterized by hypotension

and organ dysfunction [9]. Diabetics are known to be more prone to wounds and sores with poor healing, and this makes diabetics more susceptible to nosocomial infections when they are hospitalized [10].

The non-pharmaceutical approaches towards diabetic control, treatment, management, and diabetic reversal often depends on the severity of the disease, the age of the subject, their lifestyle, and their physical activity levels. The conventional clinical approaches towards diabetic control and management includes the use of sulfonylureas to increase insulin secretion; troglitazone to increase insulin action in fat and muscle cells; metformin to promote insulin mechanisms in the liver; miglitol and acarbose to delay the absorption of carbohydrates from food intake [11]. There are several concoctions that are used to treat COVID-19 patients with DM including dipeptidyl peptidase-4 (DPP-4) inhibitors, glucagon-like peptide receptor agonists, sodium-glucose co-transporter-2 inhibitors, and other analogues of insulin. But there are more novel drugs and compounds that are still required to combat DM that are cheap, and that produces low side effects for the user [12].

The medicinal potential of nature is diverse and has from the beginning of time funded the human and animal health and wellness with sources of beneficial compounds and phytonutrients without causing the negative side effects of synthetic food and drugs. The maxima advantage of plant-based remedies over synthetics is always due to their therapeutic potency versus the side-effects they cause, and the wide diversity of beneficial phytochemicals that plants contain [13, 14, 15].

According to Krakowska and colleagues, one of the most cultivated perennial fodder crop legumes in the world is *Medicago sativa* L. (also called Lucerne or Alfalfa) [16]. *Medicago sativa* L. appears in ancient medicinal herb/plant books such as *the Compendium of Materia Medica* by Li Shizhen (published .1596) and it is described in it as the father of all foods: "alfac-facah" [17].

M. sativa belongs to the legume (Fabaceae) family; it is a perennial plant of 30-100 cm in height. The taproots (invaded by Rhizobium bacteria) are well developed, thick and strong and grow very deep into the soil layers [17]. The leafy stems are erect,

clumped and even supine [18]. The leaflets of *M. sativa* are 5-20 mm long and dentate (toothed) at the apex and sometimes at the base. The papilionaceous flowers of alfalfa are violet to pale lavender and are clustered along the unbranched axis.

The use of any plant or plant part for medicinal purposes indeed is very diverse and in most cases the knowledge is forbidden for the general public. The traditional use of *moriana* or *muthi* (medicine derived from nature) for healing or other uses was and is still guarded by sacred initiations and secretive hierarchy of bodies or organizations that don't formally exist. As such, the scientific exploration of herbal medicines will always be subjected to the scientific method of evidence-based enquiry. But the genus *Medicago* is part of the *Leguminosae* family that contains 83 known species. *Medicago sativa* (L.) has evidently been used for many ages in China, India, Africa, and America for medicinal purposes. The plant has been shown to be rich in saponins, flavonoids, coumarins, terpenoids, vitamins and digestive enzymes.

Our objective was to establish the *in-vitro* glucose utilization activity induced by five *Medicago sativa* L. leaf extracts in L6 and C3A cells as it has never been reported. We also wanted to confirm the cytotoxicity of our extracts using the reliable MTT assay. Our aim was to confirm the existing ethnobotanical fact that alfalfa can help reduce hyperglycaemia by increasing glucose utilization in the liver (C3A/HEPG2) and in the skeletal muscles (L6 myoblasts).

2. METHODOLOGY

2.1. Plant material and extraction

The test material (*M. sativa* L. leaves) was purchased from a nursery and a medicinal plant farm North of Pretoria (South Africa) with the Batch Number MH 71(10kg), sold as herbal tea indicated for diabetes, cancer treatment side effects and others. Tea is to be prepared from the herbs. The plant material was cultivated by Zizameleni Farming based in Mamogali'skraal, Northwest Province of South Africa. The test material was cultivated using regenerative natural farming principles and the fertilizers used were natural and certified organics. The material was air-dried and stored in a cool dry area away from light and heat. The dry,

grassy, and pale green leaves were extracted using five solvents: butanol, diethyl-ether, hexane, methanol and water. Sixty (60) grams of powdered plant material was extracted in 1000 mL of each solvent, and the extracts were then filtered using a Buchner funnel and Whatman No.1 filter paper. The extracts were frozen at -40 °C and freeze-dried for 48 hours that resulted in a yield of 9 g of dried extracts. The dried extracts were stored at -4 °C until analysis. Dimethyl sulfoxide (DMSO) was used to reconstitute the extracts prior to analysis.

2.2. Cell lines, media, reagents and assay kits

The cells HepG2/C3A and L6 myoblasts were sourced from Highveld Biological, South Africa. Eagle's minimum essential medium (EMEM), 2-(4, 5-dimethylthiazol-2-yl) (MTT)-2, and 5 diphenyl tetrazolium bromide were supplied by Sigma Aldrich, South Africa. Fetal calf serum (FCS) and phosphate-buffered saline (PBS) were supplied by Lonza Biologics. The other materials and reagents used for the analysis were sourced either from Merck Chemicals, or Sigma Aldrich.

2.3. Maintenance of cell cultures

The L6 cells were routinely grown in low glucose Dulbecco's modified eagle medium (DMEM); they were then supplemented with 10% Fetal calf serum (FCS) and sub-cultured by trypsinisation. HepG2/C3A cells were grown in EMEM supplemented with 10% FCS and sub-cultured by trypsinisation.

2.4. Cytotoxicity evaluation assay

At the end of the glucose utilisation assay, all the remaining medium was removed. 100 µl of fix solution (0.4% formaldehyde in PBS) was added to each well and the plate was incubated at room temperature for 1 hour. After the removal of the fix solution, 100 µl of crystal violet (0.1%) was added and the plate was incubated at room temperature again for 20 minutes. The plates were washed with water to remove excess unbound dye and plates were then dried overnight at 37 °C. 100 µl of acetic acid was then added to each well and the absorbance was read at 570 nm using the BioTek® SYNERGY Mx fluorometer (Winooski, VT, USA). The cytotoxicity is expressed as a percentage of the untreated control.

2.5. The glucose utilization activity testing of *Medicago sativa* L (alfalfa) in L6 and HepG2/C3A cells

2.5.1. Glucose utilization in L6 cell assay procedure

The L6 cells were seeded into 96-well plates at a density of 5000 cells/well. At least one row was kept devoid of cells to represent the no cell control. At approximately 80% confluence, the medium was replaced with differentiation medium (DMEM containing 2% horse serum). The cells were then incubated for an additional 5 days to allow sufficient time for full differentiation.

The treatment was continued for 48 h. The spent culture medium was then removed, and the cells were washed once with phosphate-buffered saline (PBS). Fifty micro-litres of 8 mM glucose solution (Roswell Park Memorial Institute medium (RPMI) diluted with PBS and supplemented with bovine serum albumin (BSA) to a final concentration of 0.1%) was added. For the positive control, 1000 ng/ml insulin was used. The plates were returned to the incubator. After 2 hours 5 µl culture medium was transferred to a new 96-well plate and 200 µl glucose assay reagent (glucose oxidase/peroxidase colorimetric reagent) was added and the plate was incubated again at 37 °C for 10-20 minutes. The absorbance was measured at 510 nm using the BioTek® SYNERGY Mx fluorometer (Winooski, VT, USA). Glucose utilisation was calculated as the difference between the mean of the no cell wells and the test sample and expressed as a percentage of the untreated control.

2.5.2. Glucose utilization in HEPG2/C3A cell assay procedure

The HEPG2/C3A cells were seeded into 96-well plates at a density of 5000 cells/well. At least one row was kept devoid of cells to represent the no cell control. Cells were incubated for an additional 2-3 days to become confluent. The spent culture medium from differentiated cells was then removed and replaced with fresh medium containing test compounds at the concentrations of 200 µl/well. Metformin was used as positive control. The treatment was continued for 48 h. Spent culture medium was then removed, and the cells were washed once with PBS. Fifty micro-litres of 8 mM glucose solution (RPMI medium diluted with PBS and supplemented with BSA to a final concentration

of 0.1%) was added. The plates were then returned to the incubator. After 3 hours 5 µl was transferred into a new plate and 200 µl glucose assay reagent (glucose oxidase/peroxidase colorimetric reagent) was added and incubated at 37 °C for 10-20 min. Absorbance was measured at 510 nm using the BioTek® SYNERGY Mx fluorometer (Winooski, VT, USA). Glucose utilisation is calculated as the difference between the mean of the no cell control and the test sample and expressed as a percentage of the untreated control.

3. RESULTS

The results of studies measuring glucose absorption in HEPG2/C3A cells using five leaf extracts of *Medicago sativa* L. (butanol, diethyl-ether, hexane, methanol, and water) as well as metformin as the control are shown in Figure 1. The glucose utilization observed in HEPG2/C3A cells was considerably uniform but the water extract at 50 µg/mL produced glucose use almost similar to our control metformin (Figure 1). The cytotoxicity potential of the plant materials that could be used for drug development is important to ensure the consumer's safety, and our experiments demonstrated no evidence of significant cytotoxicity towards differentiated HEPG2/C3A cells.

Figure 2 displays the outcomes of cytotoxicity testing and glucose uptake of five leaf extracts of *Medicago sativa* L. (butanol, diethyl-ether, hexane, methanol, and water) using L6 myoblast cells. Metformin was employed as the control. The glucose utilization observed in L6 myoblast cells was considerably uniform but butanol and diethyl-ether produced glucose use almost similar to our control insulin (Figure 2). The cytotoxicity potential of the plant materials that could be used for drug development is important to ensure the consumer's safety, and our experiments demonstrated no evidence of significant cytotoxicity towards differentiated L6 cells.

4. DISCUSSION

From our experiments we observed the Butanol and Diethyl-ether extracts producing a sizable increase in glucose utilisation in skeletal L6 cell line (Figure 2), whereas the diethyl-ether and water extracts revealed significant increase in glucose utilisation towards the hepatic C3A cell line (Figure 1).

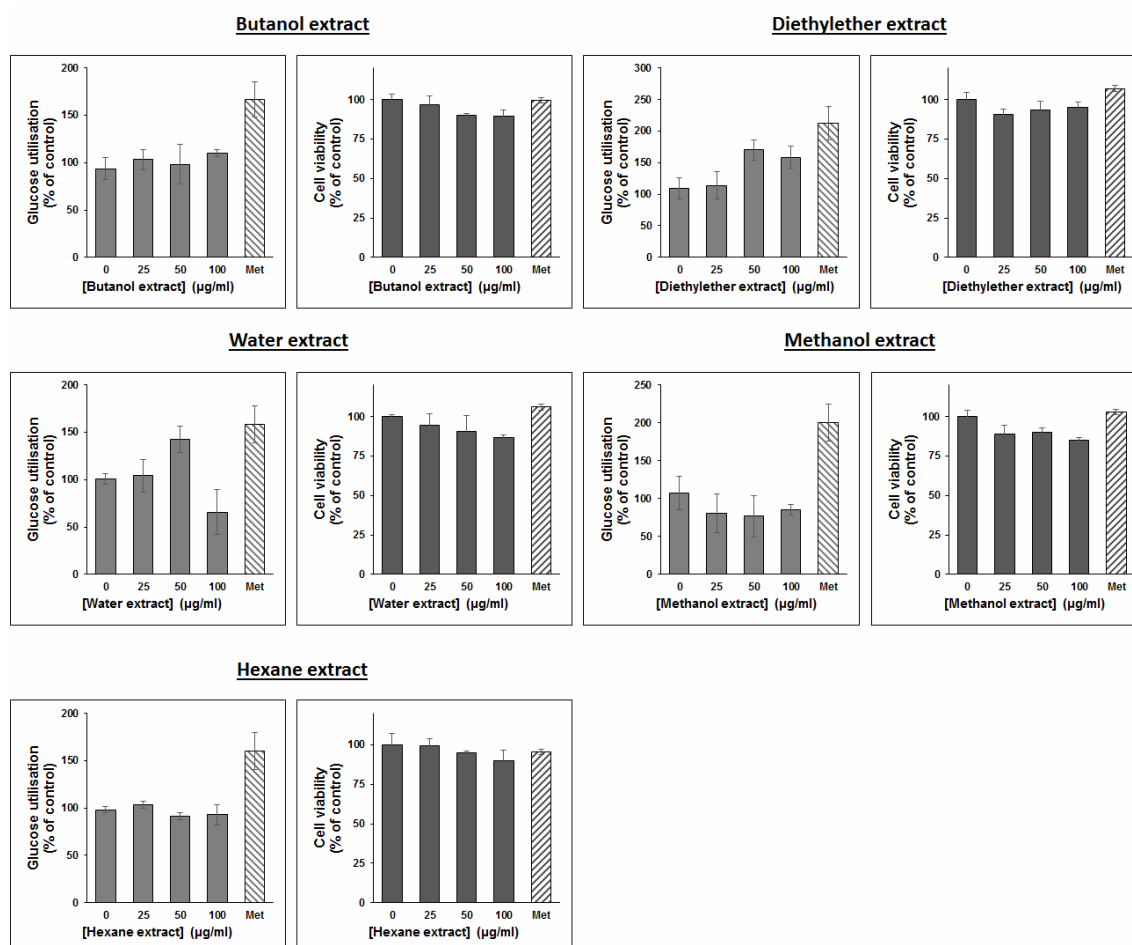


Figure 1. Glucose utilisation and corresponding cytotoxicity towards C3A/HEPG2 cells. Data is expressed as a percentage of the untreated control and metformin included as a positive control.

The overall cytotoxicity test was weak and is unlikely to have any meaningful physiological impact (Figures 1 and 2). The extracts did not induce potent activity (glucose utilization) compared to the control's insulin and metformin, which are well used in clinical setting to treat cystic fibrosis, DM, and others.

To measure the glucose utilization bioactivity induced by our five *Medicago sativa* L. extracts using liver cell line C3A/HEPG2 we used metformin as a positive control (Figure 1). The cell line C3A is a clonal derivative of HEPG2 cell line that was selected due to its strong contact inhibition of growth, high albumin production, high production of alpha fetoprotein (APF) and the ability to grow

in glucose deficient medium. The corresponding cytotoxicity towards C3A cells is shown in Figure 1, where data is expressed as a percentage of the untreated control. And according to the results only the diethyl ether and water extracts demonstrated significant increase in glucose utilisation relative to metformin; however we are not saying other extracts did not induce increased glucose use. In this context, increased glucose utilization by *Medicago sativa* could subsequently lead to the decrease of glucose in the blood; that is to say it alleviates hyperglycemia but only in laboratory conditions.

Glucose is a six-carbon structure (C₆H₁₂O₆) that is bio-processed for the generation of energy that

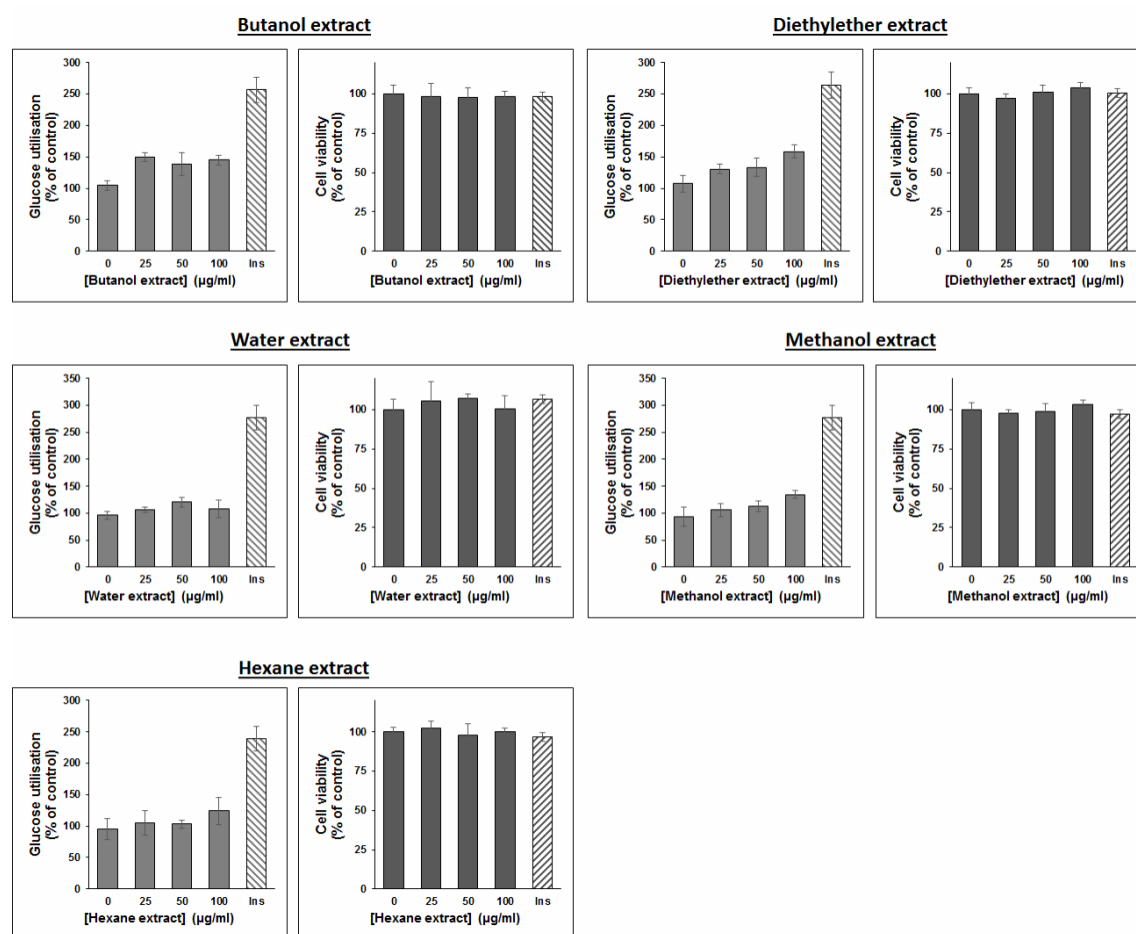


Figure 2. Glucose utilisation and corresponding cytotoxicity towards L6 skeletal muscle cell line. Data is expressed as a percentage of the untreated control and insulin employed as a positive control.

is required by organisms to function. Glucose is consumed *via* several pathways and processes that ultimately convert it to energy in the form of adenosine triphosphate (ATP). Glucose homeostasis is therefore an essential action of maintaining the energy levels of the body in the form of glucose that is circulating in the blood or that is stored in the liver in the form glycogen. The feedback mechanism that regulates blood glucose levels depends on the effective and efficient functioning of the hormone insulin.

The ancient Egyptian ‘*Chief of Dentists and Physicians*’, Hesy-Ra was the first in 1552 B.C. to describe the well-known symptom of diabetes which is frequent urination in the ancient book called

Ebers papyrus. The earlier ‘diagnostic tests’ for DM involved the observations of abnormally frequent urination, the tasting of urine for sweetness and what I call the ant-urine-attraction test/assay (where the positive diabetes test = ants being attracted to urine). The combination of these observations helped to create the name of the condition DM where mellitus means honey (sweetness), and diabetes refers to a process of siphoning (frequent urination).

Human insulin (or insulin human) is a drug synthesized through DNA recombinant techniques, to control and regulate the rate of glucose and amino acid cellular uptake. Insulin is injected subcutaneously to affect the blood glucose levels

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in type 1 and type 2 DM. The common side effects of insulin therapy include excessive hypoglycaemia, weight gain, gut discomfort, nausea, and the usual organ failure (liver and heart).

There are many other therapeutic tools used to manage and control DM, such as the use of microcapsules that carry pig-derived islets of Langerhans, which are then implanted beneath the skin of a diabetic patient to restore glucose homeostasis. Metabolic syndrome is a complex mix of abnormalities that can include obesity, insulin resistance (IR), hypertension, glucose intolerance and dyslipidaemia [19].

Obesity often disrupts the Krebs's Cycle and as a result can cause mitochondrial dysfunction which has been established to promote excessive production of ROS. Adipocytokines in obese individuals are also implicated as the cause of oxidative stress and

inflammation, where they can promote insulin resistance and thus induce type-2 diabetes (non-insulin dependent diabetes) and cardiovascular diseases [7]. Insulin resistance (IR) refers to a metabolic state where the body's ability to utilize glucose is impaired due to a decreased or abnormal response to insulin and decreased insulin production [8].

The oral treatment with water extracts of *Terminalia paniculata* bark, demonstrated a decreased blood glucose and glycosylated haemoglobin levels in streptozotocin-nicotinamide-induced diabetic rats [20]. The experiment further established the *in-vivo* inhibition of the enzymes pancreatic alpha-amylase and alpha-glucosidase. The chemical profiling of *Terminalia paniculata* using high performance liquid chromatography (HPLC) indicated the presence of bioactive biomarkers gallic acid, ellagic acid, catechin and epicatechin [20].

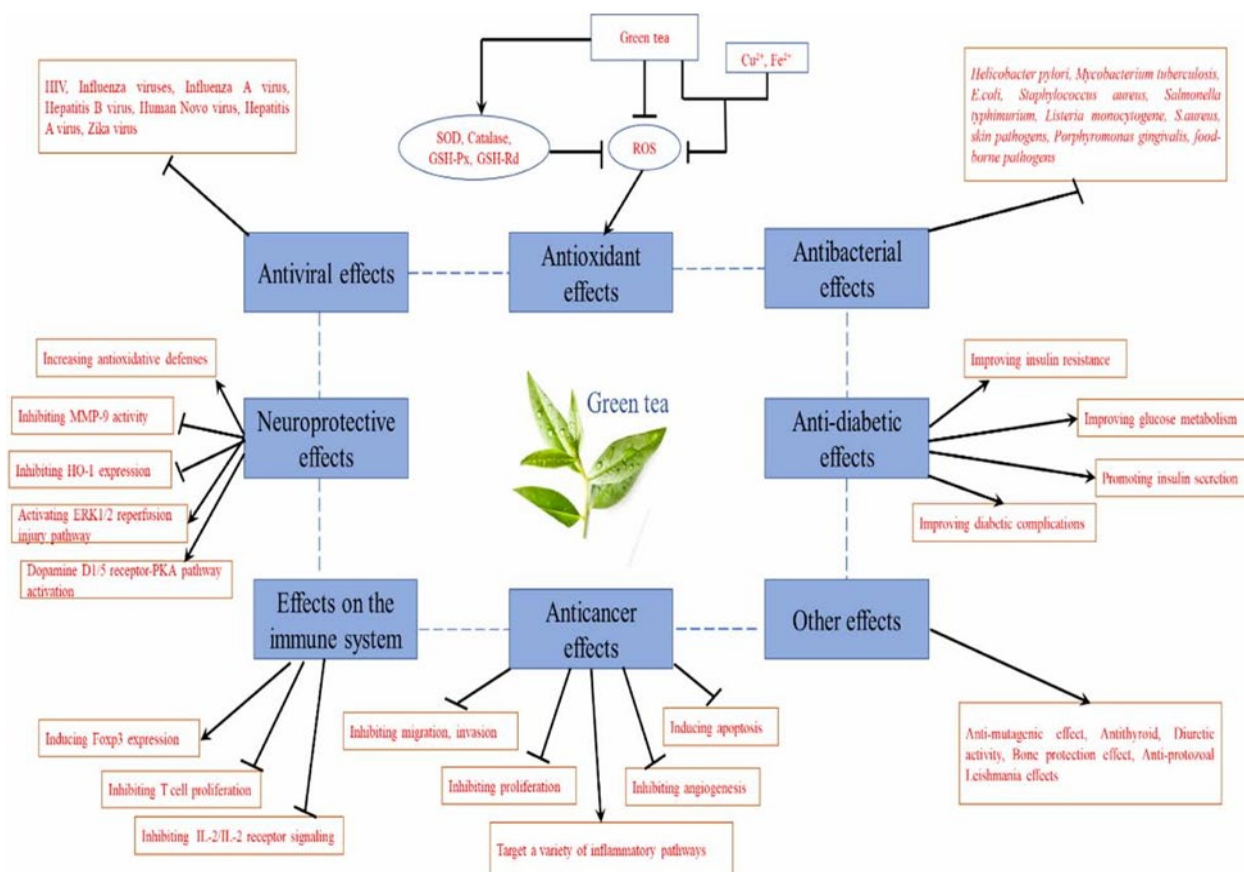


Figure 3. Pharmacological effects of green tea [21] (<https://www.mdpi.com/1420-3049/27/12/3909/htm>).

The active catechins (epicatechin gallate, epigallocatechin gallate) contained in green tea were demonstrated to inhibit the activity of glucose transporter 1 (GLUT-1) which is excessively expressed in cancer cells. This observation indicated the mechanism by which tea polyphenols can act as competitive inhibitors of glucose uptake through binding to GLUT-1 (Figure 3) and as thus induce anti-diabetic effects.

Our objective with the experiment was to determine and report the *in-vitro* glucose utilization activity of *Medicago sativa* L. leaf extracts towards L6 and C3A cell lines using five different solvents. The aim was to use the results to validate the existing evidence of anti-diabetic effects of drinking alfalfa tea.

5. CONCLUSION

Diabetes is a global concern that threatens the competence of modern medical care. To curb the emergent exponential rise of DM prevalence, affordable solutions are needed urgently. The sensitivity of contextualizing experimental results is explained by David Klatzow in his book '*Justice denied*', where he argues that the detection of opiates in a test sample for example, can lead to erroneous conclusions if we ignore that the test subject ate a roll of bread with poppy seeds sprinkled on top before the test. Thus, the spiritual aspects of traditional and African healing are also difficult to measure and to record using empirical methods.

Our experiments demonstrated clearly that every one of our five extracts we tested did impose a considerable glucose utilization enhancement both at the liver (C3A cell line) and skeletal muscle level (L6 cell line). We admit that our test extracts did not produce the bioactivity that is as strong as the activity induced by the clinical standards that we used (metformin and insulin). Our experimental results can corroborate the religious drinking of alfalfa tea for health and wellness. The result of our experiments hereby confirms the ethnobotanic and traditional uses of alfalfa towards the control of hyperglycaemia. *Medicago sativa* L. appears in ancient medicinal herb/plant books such as the *Compendium of Materia Medica* by Li Shizhen and it is described in it as the father of all foods: "*alfac-facah*" [17]

and our experiments can be used to scientifically to validate that opinion. Our recommendation is further analysis at molecular level to establish the possible mechanisms of action exhibited by alfalfa and further isolation and testing of the active compound(s) of interest.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST STATEMENT

We confirm no conflict of interests.

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APPENDIX B: MANUSCRIPT BRAVO



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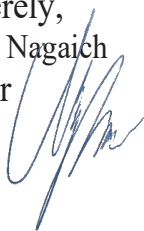
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Original Article

In-Vitro bioactivity testing of *Medicago sativa* L. leaf for anti-microbial, and cytotoxicity screening against Vero cells

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ABSTRACT

Anti-Microbial Drug Resistance (AMR) in pathogenic microbial organisms is a major threat to global public health. A certain study. isolated and tested a phenyl-propanol derivative from *Tabernaemontana incospicua* Stapf. (Apocynaceae) that not only displayed significant antimicrobial effects against the infectious *Haemophilus influenzae* 9435337A with the Minimum Inhibitory Concentration (MIC) of 62.5 µg/mL but also provides potential proof-of-ability towards combating the AMR pandemic using medicinal plants. In-vitro anti-microbial activity testing of five Alfalfa leaf extracts was evaluated using the Broth Micro-Dilution Assay and the colorimetric Microplate Alamar Blue Assay (MABA). Clinical antimicrobials Gentamicin, Vancomycin, and Fluconazole were used as controls. Cytotoxicity assays were used to determine whether cells continue to proliferate after exposure to a test compound for a specific time. The 3,4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to assess Vero cell viability following exposure to five *Medicago sativa* L. extracts. Vero cells treated with hexane, diethyl ether, and water extracts induced no decrease in the total number of cells compared to the untreated control. However, an increase of dead cells was observed after methanol, and butanol extracts treatment at 200 µg/mL. The global impact of AMR is wide and adverse, causing extended hospitalizations that amount to higher medical bills and high mortality rates.

Keywords: Anti-microbial drug resistance (AMR), Minimum inhibitory concentration (MIC), Microplate alamar blue assay (MABA), 3,4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT)

Introduction

Pharmaceutical research and development continue to struggle to meet the clinical needs of novel and effective anti-microbial agents during the ever-evolving anti-microbial drug resistance pandemic [1, 2]. The last two decades have seen the development of two novel antibiotic classes against Gram-positive bacteria: lipopeptides and oxazolidinones [3]. But the deficient existing library of newly approved anti-microbial compounds motivates

an urgent need for testing natural medicines with anti-microbial activity against pathogenic and drug-resistant micro-organisms. While superbugs (Antimicrobial Drug-Resistant Pathogens) kill over 700 000 people a year [4], the insect world also possesses potential bioactivity against biofilms as in the use of the degrading enzymes derived from the rat-tailed maggot of the drone fly *Eristalis tenax* [5]. The production of harmonine by the harlequin ladybird (*Harmonia axyridis*) is another example of a potential nature-derived antimicrobial agent used against malaria and tuberculosis as demonstrated by Christian Rohrich and others [6].

The Gram-negative organisms are notoriously implicated in

mostly fatal intra-abdominal infections, urinary tract infections, pneumonia, and bacteraemia. The strong outer membrane of Gram-negative pathogens is responsible for the globally observed antibiotic resistance [7]. The innovation and development by Zurich University and Polyphor; the so-called Outer Membrane Protein Targeting Antibiotics (OMPTA), is a novel class of

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chimeric, cationic, and peptidomimetic antibiotics that have since demonstrated therapeutic activity against antibiotic-resistant Gram-negative pathogens [7].

The Anti-microbial resistance pandemic is a global concern affecting both developed and developing countries. There is no alternative but to control the use of antimicrobial drugs, to interpret the mechanisms of resistance, and thus to engineer novel therapeutics against drug-resistant pathogens [8]. The need for effective and cost-friendly therapeutics is urgent and requires a multi-pronged approach that includes looking back into our past (*Sankofa*) for clues and solutions [8]. The use of plants and plant-derived agents for medicinal purposes by humans and animals is old, diverse, and advanced. For humans and animals, plants play a central role in their health and wellness, vitality, and survival as plants continue to supply a large share of beneficial compounds and molecules to the food and Agri-pharmaceutical industries.

The most common bacterial Urinary Tract Infection (UTI) in women is caused by *Escherichia coli* [9]. The infection can occur anywhere in the urinary tract but frequently affects the bladder, urethra, or kidneys [9]. Antibiotic drugs are invaluable in the management and treatment of infections but also in the decrease of deaths associated with infections [10]. At the same time, *Enterococcus faecalis* is responsible for several nosocomial infections of the bloodstream, urinary tract, and of surgical sites [11]. Bacterial Meningitis (caused by *Streptococcus pneumoniae*) is a life-threatening condition that can lead to brain damage with sensual and cognitive impairment [12]. The species of *Candida* are notorious in the bloodstreams of hospitalized patients, as well as generally infecting our mouths, skins, and vagina [13]. *S. epidermidis* is also well-known as a frequent cause of prosthetic joint infections, prosthetic valve endocarditis, and neonatal sepsis [14].

Anti-biotic susceptibility testing is an effective tool in the treatment and management of infections; it allows clinicians to select the appropriate anti-microbial agent and to prescribe with precise accuracy, the bactericidal/bacteriostatic dosage [15]. The prescribed anti-biotic needs to be effective without causing significant or severe negative side effects to the person or animal or plant. The application of anti-microbial susceptibility testing for the evaluation of the potential bioactivity of natural products is one of the effective interventions instigated to curb the current surge of global AMR [16].

Cytotoxicity assays are tools used to determine whether cells continue to proliferate after exposure to a test compound for a specific time. Cytotoxicity is usually assessed by methods that compare either the cellular (enzymatic) function and/or cell integrity of control cells to those exposed to a test compound [17]. We used a quantitative colorimetric assay based on the metabolic activity of cells to evaluate cell viability after exposure to Alfalfa extracts (MTT assay).

This paper aimed to evaluate the anti-microbial bioactivity in-vitro and to report the MIC of Alfalfa (*Medicago sativa* L.) leaf extracts against a panel of five clinical pathogenic strains: *E. coli*, *E. faecalis*, *S. epidermidis*, *S. pneumoniae*, and *C. albicans* using the Broth Micro-dilution method and Microplate Alamar Blue assay.

Materials and Methods

The test (plant material), the extraction, and the preparation for in-vitro bio-analysis

The plant material (dried *M. sativa* L. leaves) was purchased from a nursery and medicinal plant farm North of Pretoria (South Africa) with Batch Number MH 71 (10kg) as shown in **Figure 1**. The plant material was cultivated by Zizameleni Farming based in Mamogaleskraal, Northwest Province of South Africa. The test material was cultivated using regenerative natural farming principles and the fertilizers used were all natural and certified organics. The material was air-dried and stored in a cool dry area away from light and heat. The dry, grassy, and pale green leaves were extracted using five solvents: Butanol, Diethyl-ether, Hexane, Methanol, and Water. Sixty (60) grams of powdered plant material were extracted in 1 litre (1000 mL) of every solvent. The extracts were filtered using the Buchner funnel and Whatman no.1 filter paper. The extracts were frozen at -40 °C and freeze-dried for 48 hours at a yield of 9 g of dried extracts. The dried extracts were stored at -4 °C until analysis.



Figure 1. *M. sativa* L. leaves

The in-vitro bioactivity testing of five Alfalfa leaf extracts against pathogenic micro-organisms

There are countless approaches and techniques employed for the screening of biological extracts for therapeutic potential use as antimicrobial agents. The Broth Micro-dilution susceptibility method in a 96-well microtiter plate has become the preferred method for drug susceptibility testing due to its small sample requirements and high-throughput rate [18]. Microplate Alamar Blue assay (MABA) is a colorimetric assay that can be utilized for drug-susceptibility testing based on oxidation/reduction reactions [19]. This assay uses an oxidation/reduction indicator; Alamar Blue (CellTiter-Blue®), that changes color from blue to pink, indicating bacterial growth. The color change can be read visually and quantified fluorometrically by excitation at 530 nm and detecting emission at 590 nm [19].

Materials for the anti-microbial bio-activity testing of Alfalfa extracts

Enterococcus faecalis, *Staphylococcus epidermidis*, and *Escherichia coli* (clinical strains) were grown in Mueller-Hinton (MH) broth

(Merck, USA). *Candida albicans* were maintained in Malt Extract broth (Merck, USA). *S. pneumoniae* was grown in Brain-Heart Infusion broth (Merck, USA). One microorganism colony, from an overnight streak plate, was inoculated in broth (10 mL) and allowed to grow for 16 hours (log growth phase) at 37 °C. Control drugs used: Gentamicin sulfate and Vancomycin hydrochloride (Sigma, USA) were used as positive controls against Gram-negative and Gram-positive bacteria, respectively. Fluconazole (Sigma, USA) was used as a positive control against *C. albicans*. Antibiotics were dissolved in ddH₂O at stock concentrations of 2 mg/mL and filter sterilized (0.2 µm filter). Working concentrations were prepared in broth, depending on the antibiotics' MIC values. Test Extracts were dissolved in Dimethyl Sulfoxide (DMSO) at stock concentrations of 100 mg/mL. Working concentrations of 4 mg/mL were prepared in MH broth/Malt Extract/Brain-Heart infusion broth.

The Anti-microbial bio-activity testing procedure (broth micro-dilution assay)

50 µL of MH broth/Malt Extract/Brain-Heart infusion broth was added to all test wells (i.e., plant extracts and antibiotics), except for the highest plant extract and antibiotic concentration wells to which 100 µL of the working concentrations were added. Serial dilutions were prepared for the plant extracts: 2 mg/mL to 125 µg/mL and for antibiotics: 64 to 0.25 µg/mL. The cultures were assessed and adjusted to a 0.5 McFarland standard (absorbance at 600 nm = 0.08-0.1; equivalent to ~1.5x10⁸ cells/mL) and 50 µL was added to each test well.

Preparation of Controls:

Antibiotic/medium control (50 µL broth + 50 µL of highest antibiotic concentration). Extract color control (50 µL broth + 50 µL of highest plant extract concentration). 4% DMSO control (50 µL broth + 50 µL 8% DMSO). Microorganism control (50 µL broth + 50 µL microorganism). Plates were sealed with microplate sealing tape and incubated at 37°C for 24 h.

CellTiter-Blue® assay

After treatment, 20 µL of CellTiter-Blue® Reagent (Promega) was added to each well and incubated for 1 h. Wells were observed for the color change, where a blue color represented no growth (non-viable cells) and a pink color represented growth (viable cells). The fluorescence was read at excitation and emission wavelengths of 535 and 590 nm, respectively, using a BioTek® SYNERGY Mx fluorometer (Winooski, VT, USA).

The in-vitro cell viability testing of five Alfalfa leaf extracts against vero cells

The 3,4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is commonly used to assess cell viability. The MTT assay is a quantitative colorimetric assay based on the metabolic activity of cells. The yellow water-soluble tetrazolium dye is reduced to a dark blue-to-purple insoluble formazan

product by mitochondrial enzymes of viable cells. The insoluble formazan product is dissolved in dimethyl sulfoxide (DMSO) and can be measured spectrophotometrically at 540 nm. The amount of product produced is proportional to the number of viable cells [20, 21]. The limitation of this method is that other factors can influence the metabolic activity of cells and therefore we cannot accurately measure cell death [20].

Measuring the integrity of cell membranes is an alternative reliable method that can be used to assess cell viability [20] Dual staining with Hoechst and Propidium iodide (PI) has been used to assess viable and nonviable cells, respectively. Hoechst 33342 is a membrane-permeant dye that binds to the minor grooves of double-stranded DNA. Hoechst 33342 is excited at a wavelength of 350 nm and emits a blue fluorescence at 461 nm. Because Hoechst is a cell-permeant dye it stains all nucleic acids and therefore gives a total cell count, i.e., live plus dead cells [22]. PI is a red fluorescent molecule that binds to DNA and is excited at 536 nm and emits light at 623 nm. PI uptake is dependent on the integrity of the cell membrane and cannot enter cells with intact membranes. The uptake and exclusion of this dye can therefore be used to discriminate between viable and nonviable cells [23, 24].

Materials and methods

Vero cells were purchased from Cellonex, South Africa. DMEM low glucose cell culture medium and FBS were purchased from GE Healthcare Life Sciences (Logan, UT, USA). PBS with and without Ca²⁺ and Mg²⁺ and trypsin-EDTA was purchased from Lonza (Walkersville, MD, USA). Bis-benzamide H 33342 trihydrochloride (Hoechst) and propidium iodide (PI) was purchased from Sigma (St. Louis, MO, USA).

Image acquisition and analysis

Images were acquired using the ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices). A plate acquisition setup for a 96-well plate at a magnification of 10 times was used. Spatial distribution for the acquisition was set at 9 sites per well (3 by 3). The 9 images per site cover roughly 70% of the total well area. Filters used included DAPI (for Hoechst) and Texas Red (for PI). Analysis of acquired images was performed using the Multi-Wavelength Cell Scoring analysis module of the MetaXpress version 6.1 High-Content Image Acquisition and Analysis Software.

Cell seeding and treatment

Vero cells were seeded at a density of 5000 and 3000 cells/100 µL/well and incubated overnight at 37°C for attachment. Treatment for cytotoxicity assay was done by adding 100 µL of extract to achieve final concentrations of 50, 100, and 200 µg/mL. Cells were incubated at 37°C for 48 h. Melphalan at 6.25, 12.5, and 25 µg/mL served as the positive control as shown below in **Table 1**.

Cytotoxicity testing of Alfalfa leaf extracts

procedure

After 48 h treatment medium was removed. Hoechst 33342 was diluted in 10 mL DPBS with Ca²⁺ and Mg²⁺ to a final

concentration of 5 µg/mL and added to wells using 100 µL aliquots. Cells were incubated at 37°C for 30 min. PI was diluted in 1 mL PBS to a concentration of 110 µg/mL and added to wells in 10 µL aliquots before image acquisition to achieve a final concentration of 10 µg/mL.

Table 1. The Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	[50] rep1	[100] rep1	[200] rep1	[50] rep1	[100] rep1	[200] rep1	[50] rep1	[100] rep1	[200] rep1	[50] rep1	[100] rep1	[200] rep1
B	[50] rep2	[100] rep2	[200] rep2	[50] rep2	[100] rep2	[200] rep2	[50] rep2	[100] rep2	[200] rep2	[50] rep2	[100] rep2	[200] rep2
C	[50] rep3	[100] rep3	[200] rep3	[50] rep3	[100] rep3	[200] rep3	[50] rep3	[100] rep3	[200] rep3	[50] rep3	[100] rep3	[200] rep3
D	[50] rep4	[100] rep4	[200] rep4	[50] rep4	[100] rep4	[200] rep4	[50] rep4	[100] rep4	[200] rep4	[50] rep4	[100] rep4	[200] rep4
E	[50] rep1	[100] rep1	[200] rep1									
F	[50] rep2	[100] rep2	[200] rep2	UNTREATED CONTROL			POSITIVE CONTROL: Melphalan): 6.25, 12.5, 25 µM			UNTREATED CONTROL		
G	[50] rep3	[100] rep3	[200] rep3									
H	[50] rep4	[100] rep4	[200] rep4									
A												
B	Hexane (SUNTB 1)			Diethylether (SUNTB 4)			Methanol (SUNTB 6)			Butanol (SUNTB 7)		
C	LABEL: 1			LABEL: 2			LABEL: 3			LABEL: 4		
D												
E												
F	Water (SUNTB 10)			UNTREATED CONTROL			POSITIVE CONTROL: Melphalan): 6.25, 12.5, 25 µM			UNTREATED CONTROL		
G	LABEL: 5											
H												

Results and Discussion

Results of the screening of five extracts of *Medicago sativa* L. leaves for antimicrobial activity against a panel of five microorganisms (*E. coli*, *E. faecalis*, *S. epidermidis*, *S. pneumoniae*, and *C. albicans*), using the Broth Micro-dilution method and Microplate Alamar Blue assay

Anti-microbial drug resistance (AMR) in pathogenic bacteria is a global concern that is threatening the effectiveness of conventional clinical care systems and treatments. The World Health Organization (WHO) considers AMR “an urgent threat that requires a multisectoral action to achieve the Sustainable Development Goals (SDGs)” [25]. ARM also imposes a pressurizing force on major medical interventions such as surgery and cancer chemotherapy [26]. The poor service delivery to disadvantaged communities and villages in developing countries means a lack of water and poor sanitation which can affect infection control and prevention.

The Broth Micro-Dilution Assay was used to evaluate the anti-microbial activity of *Medicago sativa* L. leaves against five clinical

strains (*E. coli*, *E. faecalis*, *S. epidermidis*, *S. pneumoniae*, and *C. albicans*). The method is favored for its highly accurate results, availability of testing plates and standard reagents, and the possibility of reading results both qualitatively (color changes) and quantitatively by Spectrophotometric techniques [27].

The experimental findings establish a scientific fact that plants can inhibit the growth and multiplication of disease-causing micro-organisms. This finding gives the religious habit of drinking herbal tea for health and wellness more value and potentiates the use of *Medicago sativa* L. as a possible alternative non-drug anti-microbial agent.

Results of cytotoxicity screening of Alfalfa Extracts in vero cells

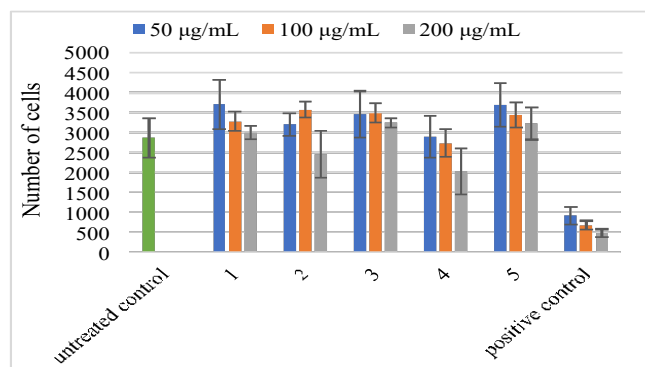


Figure 3. Total number of cells (live + dead) after treatment with extracts and positive control (melphalan).

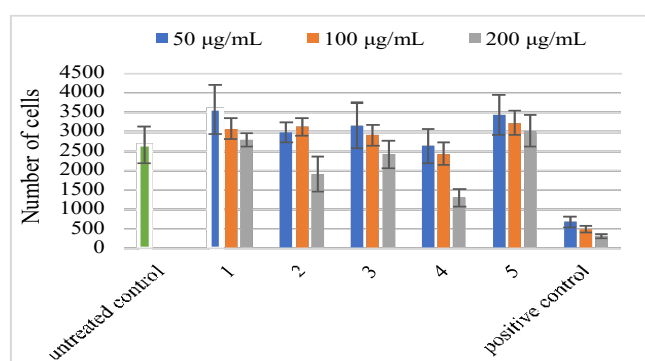


Figure 3. Number of live cells after treatment with extracts and positive control (melphalan).

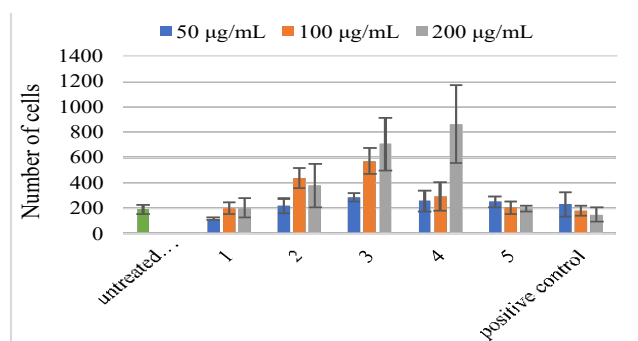


Figure 4. Number of cells with PI positive staining (dead cell number) after treatment with extracts and positive control (melphalan).

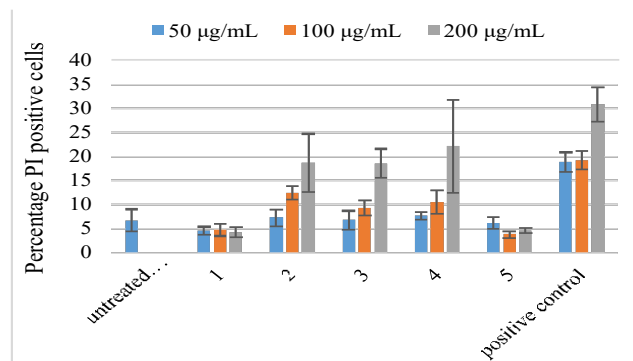


Figure 5. Percentage PI positive after treatment with extracts and positive control (melphalan).

Cytotoxicity assays are tools used to determine whether cells continue to proliferate after exposure to a test compound for a specific time. Cytotoxicity results are shown and discussed for a cell density of 5 000 cells/100 µL/well. The Total number of cells (live + Dead): Vero cells treated with hexane (1), methanol (3), and water (5) extracts did not show a decrease in the total number of cells compared to the untreated control (**Figure 2**). A decrease in the total number of cells was observed for diethyl ether (2) and butanol (4) extracts at 200 µg/mL (**Figure 2**). The Number of Live cells: Vero cells treated with hexane (1) and water (5) extracts did not show a decrease in the number of live

cells compared to the untreated control (**Figure 3**). A decrease in the number of live cells was observed for diethyl ether (2), methanol (3), and butanol (4) at a concentration of 200 µg/mL compared to the untreated control (**Figure 3**). The Number of PI-positive Cells (dead cells): Vero cells treated with hexane (1) and water (5) did not show an increase in the number of PI-positive cells (**Figure 4**). Diethyl ether (2), methanol (3), and butanol (4) showed an increase in the number of PI-positive cells (**Figure 4**). The Percentage PI positive: Vero cells treated with hexane (1) and water (5) did not show an increase in the percentage of PI-positive cells (6. Diethyl ether (2), methanol (3), and butanol (4) showed a dose-dependent increase in the percentage of PI-positive cells (**Figure 5**). We report therefore report that the hexane and water extracts demonstrated no evidence of toxicity or cytostatic activity. While the diethyl ether, methanol, and butanol extracts demonstrated toxicity at 200 µg/ml but not lower concentrations.

Plants and other natural sources continue to provide a diverse variety of compounds, analogues, and derivatives that can be used to formulate and develop the much-needed anti-microbial drugs that can combat antibiotic-resistant pathogens [29]. Anti-microbial susceptibility testing of medicinal plants is not only used to confirm the ethnobotanical literature but also to evaluate the plant as a prospective anti-microbial agent against drug-resistant clinical pathogens. The use of *alfac-facah* for medicinal purposes includes use as a neuroprotective, anti-microbial, anti-cancer, and cholesterol-lowering agent [28]. Newly isolated triterpenoid saponins from *M. sativa* L. demonstrated neuroprotective potential against hydrogen peroxide-induced SH-SY5Y (Human Neuroblastoma) cell death compared to the

hydrogen peroxide-treated group [28]. The toluene and methyl tert-butyl ether (MTBE) extracts of *M. sativa* L. demonstrated *in-vitro* cytotoxic effects on two murine leukemia cell lines P388, whence the extracts also induced programmed cell death by activation of the caspase-3, leading to poly ADP ribose polymerase cleavage (PARP) [29].

Conclusion

Despite the good recording of negative clinical-drug-to-herbal-drug interactions, medicinal plants continue to play a critical role in disease prevention and drug development [30]. The regular and consistent consumption of herbal infusions was a key action

that our ancestors used to flavor water and also to siphon nutrients and secondary metabolites (phytochemicals) from plants and herbs availed by nature. The native approach to processing roots and tree barks included preparation of decoctions, some fomentations for the skin, and soaking or irrigating a specific body area. The primitive hunter was also proficient in dealing with poisonous/venomous snake and insect bites, thereby using poultice or cataplasm of macerated plant material, and wrapping it against the skin or an area of much swelling probably using a leaf or flexible bark as a bandage.

A large library of the medicinal plants used for medicinal purposes still resides mostly within our traditional healers, herbalists, and medicine men/women (*li-Ngaka, Lingaka-Tjhitja, Pepeduma, Makherenkhoa, Makhehla, le Baloi*). The conventional medical care system still needs to integrate traditional or native medicinal approaches into its standard operating procedures. Advanced native Health care systems such as Ayurveda, homeopathy, Yunani Medicine, Phytotherapy, and Herbal Medicine are still largely isolated and not included in the scope of conventional medical care.

We rely on the results of our experiments reported here, to suggest that the perpetual drinking of a herbal beverage (tea) made from *Medicago Sativa/Alfalfa* leaf infusion for health and wellness purposes is hereby scientifically confirmed. Although we understand that laboratory-based conditions are far from a true reflection of the conditions *in-vivo*, we are confident to recommend the further testing of *Medicago sativa*-derived phytochemicals, as our experiments and experiments of others, demonstrate a significant therapeutic potential induced by the reliable agricultural fodder: *Medica Sativa/Lucerne/Alfac-facah*.

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finding and conclusion, or recommendation addressed by the authors of the article.

Conflict of interest: None

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Ethics statement: None

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APPENDIX C: CHARLIE MANUSCRIPT

7 July 2023

Ref. No. RT/P/390

Dear Dr. Makhoahle Pakiso Moses,

Thanks for the mail. Regarding your query, as indicated earlier, your article is scheduled for 'Current Topics in Pharmacology', Vol. 27, 2023. The release time of the print version of the volume is scheduled to be towards the end of this year. But the ahead-of-print release of articles on web would happen much before that.

For now, you may please send an 'IN PRESS' citation to your research office and inform them that the article is currently in press.

The 'IN PRESS' citation of your article is:

'Makhele Thapelo Simon, Makhoahle Pakiso Moses and Mashele Sitheni Samson, 2023, Laboratory-based anti-inflammatory and DPPH scavenging bioactivity testing of five *Medicago sativa* L. (Alfalfa) leaf extracts, Current Topics in Pharmacology, Vol. 27, IN PRESS.'

MAP kinase that can also induce the perpetual oxidative stress-inflammation cycle [3]. The modern lifestyle associated with the consumption of processed foods, increased exposure to environmental and industrial toxins and pollutants, and the lack of physical activity increases the prevalence of oxidative stress [4]. There are many negative implications of persistent oxidative stress: low grade chronic inflammation, cellular damage, steroid resistance, necrosis, epigenetic changes, and autoimmunity [5].

The nonsteroidal anti-inflammatory drug (NSAID) regularly prescribed for inflammation and pain control is diclofenac sodium (*Voltaren*) [6]. Diclofenac is associated with dose-dependent gastrointestinal, cardiovascular, and renal side effects that include gastric ulcers and bleeding, heart attack or stroke [7]. There are currently synthetic attempts to modify the chemistry of diclofenac to improve its safety profile [8].

The excess accumulation of pro-oxidants beyond oxidative-homeostatic control and regulation plays a key role in the pathogenesis of major chronic diseases [4]. The use of antioxidants both synthetic and natural is to mitigate oxidative stress and eventually the disease or the condition. There are reports of negative side effects due to prolonged use of synthetic antioxidants. For this reason, the demand for natural antioxidants such as plant-based or derived phenolics and flavonoids has increased and thus motivating the experimental evaluation of antioxidant and anti-inflammatory activities of medicinal plants such as the well-used alfalfa, both in agriculture and medicine.

The balance between pro-oxidants and antioxidants is critical for the correct physiologic functioning of bodily systems [3]. Antioxidants are molecules that interact with free radicals thus terminating the adverse chain reactions and converting them to harmless products [9]. Dr. Sandile Fuku argues that “the validity of the term “antioxidant” depends on the environment of its action (either *in vivo* or *in vitro*); that is to say that an opinion that some compound is an antioxidant *in-vitro*, may not yield any biological meaningful information” [10]. Although excess free radicals like reactive oxygen species (ROS) is a complication of many

pathologies, low levels of ROS play a major role in mitochondrial redox signalling, cell differentiation, cell migration and cell proliferation [11]. Having said this, the use of medicinal plants with antioxidative action is due to their ability to also prevent or treat a myriad of human and animal pathologies [4]. ROS can induce inactivation of signalling mechanisms between insulin receptors and the glucose transport system thereby inducing insulin resistance [4]. In diabetes, an electron transfer and oxidative phosphorylation are decoupled, resulting in the production of superoxide anions and inefficient ATP synthesis [4]. Insulin resistance (IR), abdominal obesity, atherogenic dyslipidaemia, high blood pressure, genetic predisposition and chronic stress are the main risk factors underlying the metabolic syndrome [4]. The literature indicates how several intracellular factors, including the redox state, might directly or indirectly affect the progression and outcome of viral infections [3]. The established understating that viruses can break the pro-oxidant/antioxidant equilibrium thus inducing oxidative stress, can be used as drug targets for therapies that are concerned with blocking both viral replication and viral-induced inflammation [3].

Turmeric is a strong, yellow-coloured spice and food colourant widely used in curries and has been a subject of studies both experimental and clinical for years. This spice is mainly used in food preparation for its poignant smell, hot and earthy flavouring, and taste-enhancing properties. The turmeric spice is derived from the root of the perennial, rhizomatous plant native of the Indian subcontinent and Southeast Asia called *Curcuma longa* belonging to the ginger family *Zingiberaceae* [12]. The potency of curcumin metabolites, tetrahydro-curcumin and octa-hydro curcumin was demonstrated to exert beneficial effect on LPS-stimulated inflammatory and oxidative responses through partial inhibition of NF-kB and MAPKs pathways and activation of nrf2-regulated antioxidant gene expression by Xie and others [12].

Dan-Dan Zhang and colleagues of the Shanghai University of Traditional Medicine in China demonstrated the mechanism of action of 1,3,5,7-tetrahydroxy-8-isoprenylxanthone (TIE) isolated

from a specie of *Garcinia Linn* [13]. The species of *Garcinia Linn*. are proven to be rich in natural xanthenes and benzophenones, both which bless the drug development industry with compounds that are both structurally diverse and those that impose a wide variety of proven pharmacological activities. In their well-established experiment, they established both the anti-inflammatory activity and the mechanism of action, where the extracted compounds inhibited the production of nitric oxide and prostaglandin E2 (PGE2) in LPS/IFN-gamma-stimulated RAW264.7 cells. Furthermore, they went on to show that TIE suppressed the expression of inflammatory cytokines IL-6, IL-12, and TNF-alpha: molecules that are notoriously implicated in obesity, whereby they are secreted by adipocytes which then causes the resulting inflammatory state.

Woan Sean Tan and colleagues of the Laboratory of Vaccines and Immunotherapeutics, based at the University of Putra Malaysia also demonstrated the suppression of the activation of the inflammatory mediators in LPS-stimulated RAW264.7 macrophages via the nuclear factor-kappa B (NF-kB) by the hydroethanolic extract of *Moringa oleifera* [14]. Hamza Mechachate of the Laboratory of Biotechnology at the University of Sidi Mohamed Ben Abdellah (USMBA), with collaborators based in Brazil and Finland, also demonstrated *in vitro* and also in mice the anti-inflammatory effects of the polyphenol-enriched fraction extract of *Myrtus communis* L leaves [14].

The old hunter-gatherer was blessed with access to natural compounds that are both medicinal and nutrient rich. The relationship between the African hunter and his surrounding included the ability to communicate with the honeybee (*Apis mellifera scutellata*), which guided him towards the beehive hidden deep in the bushy African forest. Propolis is a resinous compound produced by honeybees that is rich in pharmacological-active compounds that include the anti-carcinogenic caffeic acid phenethyl ester (CAPE), artemisin C, and chrysin [15]. The bioactivity of propolis is towards multiple signaling pathways including phosphoinositide 3-kinase (PI3K)/Akt and to induce cell cycle arrest. *Cannabis sativa* L. is a well-known plant containing psycho-active and pharmacological active

compounds including the well known *delta-9-tetrahydrocannabinol* or Δ -9-tetrahydrocannabinol (Δ -9-THC) and cannabidiol (CBD). Marijuana has been and is still a subject of much debate, but the positive and negatives are fenced across long term effects and the inability to return from third-eye spiritual experience.

2. MATERIALS AND METHODS

2.1. The test (plant material), the extraction and the preparation for *in-vitro* bio-analysis

The World Health Organization's global burden of disease study confirms the urgent need for therapeutics and molecules for the maintenance of health and wellness [16]. According to Krakowska *et al.* [17], one of the most cultivated perennial forage (fodder crop) legumes in the world is *Medicago sativa* L. (Lucerne or Alfalfa). *Medicago sativa* L. is listed in the ancient medicinal herbs/plant book *Compendium of Materia Medica* by the ancient medicine-man (*ngaka-tjhitja*): Li Shizhen (published in 1596) and it is described in there as the father of all foods: "*alfac-facah*" [17]. *M. sativa* belonging to the legume (*Fabaceae*) family is a perennial plant of 30-100 cm in height. The papilionaceous flowers of alfalfa are violet to pale lavender and are clustered along the unbranched axis. Lucerne is adapted to a wide variety of climatic conditions around the world thus serving as an economic crop used for animal feed production and human consumption for many generations since ancient times [18]. The test material (*M. sativa* L. leaves) was sourced from a nursery and medicinal plant farm located North of Pretoria in South Africa or Azania, not very far from the Magaliesburg Mountain ranges that form the natural barrier between the lower laying Bushveld to the north and the cooler Highveld to the south. The test material was cultivated by Zizameleni Farming based in Mamogalieskraal, Northwest Province of South Africa. The material was air-dried and stored in a cool dry area away from light and heat without chemical preservatives: Batch Number MH 71 (10KG). The material (dry leaves) is sold to the public for drinking as a hot herbal tea/ beverage. The dry, grassy, and pale green leaves were extracted using five solvents: butanol, diethyl-ether, hexane, methanol and water. The extracts were stored in a cool area away from light and heat until analysis.

2.2. The *in-vitro* bio-analysis of antioxidant potential, bio-analysis of cytotoxicity and anti-inflammatory action of alfalfa leaf extracts (butanol, diethyl-ether, hexane, methanol and water)

2.2.1. The DPPH scavenging assay, the anti-inflammatory, and cell viability (MTT) assay

2.2.1.1. Materials

Positive control stocks: Trolox and Epigallocatechin gallate (EGCG) were sourced from Sigma. DPPH: 2,2-diphenyl-1-picrylhydrazyl was also from Sigma. The two other consumables ethanol 99.9% and Tris were sourced from Sigma and Roche Diagnostics, respectively. RAW 264.7 mouse macrophages were purchased from Cellonex (South Africa). Lipopolysaccharide (LPS), Griess reagent and aminoguanidine were purchased from Sigma-Aldrich (St. Louise, MO, USA)

2.2.1.2. Assay procedure of the DPPH scavenging activity

The reagents required for the DPPH assay were prepared as follows and the 96-well plate assay was carried out as indicated in Table 1:

Tris-HCl buffer (50 mM): we dissolved 0.606 g Tris (Roche Diagnostics GmbH, Mannheim, Germany) in 80 ml of distilled water; pH was adjusted to 7.4 using HCl and then filled up to 100 ml with distilled water. DPPH (0.1 mM): we dissolved 0.002 g of DPPH in 50 ml ethanol; prepared fresh and protected from light. Extracts were tested at a range of 6.25 – 400 µg/ml.

Absorbance was measured using a Biotek® Powerwave XS microtiter plate reader (Winooski, VT, USA). The percentage of antioxidant activity (AA%) was calculated using the below formula:

$$AA\% = 100 - \left[\frac{(Abs_{sample} - Abs_{blank}) \times 100}{Abs_{control}} \right]$$

2.2.2. The anti-inflammatory and cell viability (MTT) assay

Sample preparation: Extracts were solubilised in DMSO to a final concentration of 100 mg/ml and further diluted into culture medium. Aminoguanidine (100 µM) was used as a positive control to indicate anti-inflammatory activity.

Anti-inflammatory screening protocol: RAW 264.7 cells were seeded into 96-well plates at a density of 1×10^5 cells per well and allowed to attach overnight. The following day spent culture medium was removed and the samples (diluted in DMEM complete medium) were added to give final concentrations of 25, 50, 100 and 200 µg/mL (50 µl per well at double the desired final concentration). To assess the anti-inflammatory activity, 50 µL of LPS (final concentration of 500 ng/mL) containing medium was added to the corresponding wells. Aminoguanidine (AG) was used as the positive control for this experiment. Cells were incubated for a further 18 hr. To quantify NO production, 50 µL of the spent culture medium was transferred to a new 96-well plate and 50 µL Griess reagent was added. Absorbance was measured at 540 nm, and the results were expressed relative to the appropriate untreated control. A standard curve using sodium nitrite dissolved in culture medium was used to determine the concentration of NO in each sample.

2.2.3. Cytotoxicity (cell viability) screening protocol

To confirm the absence of toxicity as a contributory factor, cell viability was assessed using methylthiazolyldiphenyl-tetrazolium bromide (MTT). This was done by the removal of the remaining medium and treatments in each well and replacing it with medium containing 0.5 mg/mL MTT and further incubated for 30 minutes at 37 °C. Thereafter, MTT was removed and 200 µL DMSO was added

Table 1. Sample volumes and steps for 96-well plate assay.

• Sample	5 µl
• Tris-HCl (50 mM, pH 7.4)	120 µl
• DPPH (0.1 mM)	120 µl
96-well plate was incubated for 20 minutes at room temperature	
Measure absorbance at 513 nm	

to each well to solubilise the formazan crystals. Absorbance was read at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA).

3. RESULTS AND DISCUSSION

Results of the DPPH scavenging activity, cytotoxicity and anti-inflammatory potential screening of five extracts of *Medicago sativa* L. leaves (Solvents: Butanol, Diethyl-ether, Hexane, Methanol and Water)

are shown in Figure 1 and Figure 2, respectively below.

Five extracts of *Medicago sativa* L. leaves were screened against RAW 264.7 cells for anti-inflammatory potential.

To determine the absence of toxicity against the cells, the MTT assay was performed and the results are shown in Figure 3.

DPPH (2,2- diphenyl-1-picryl-hydrazyl-hydrate) free radical scavenging method is an antioxidant

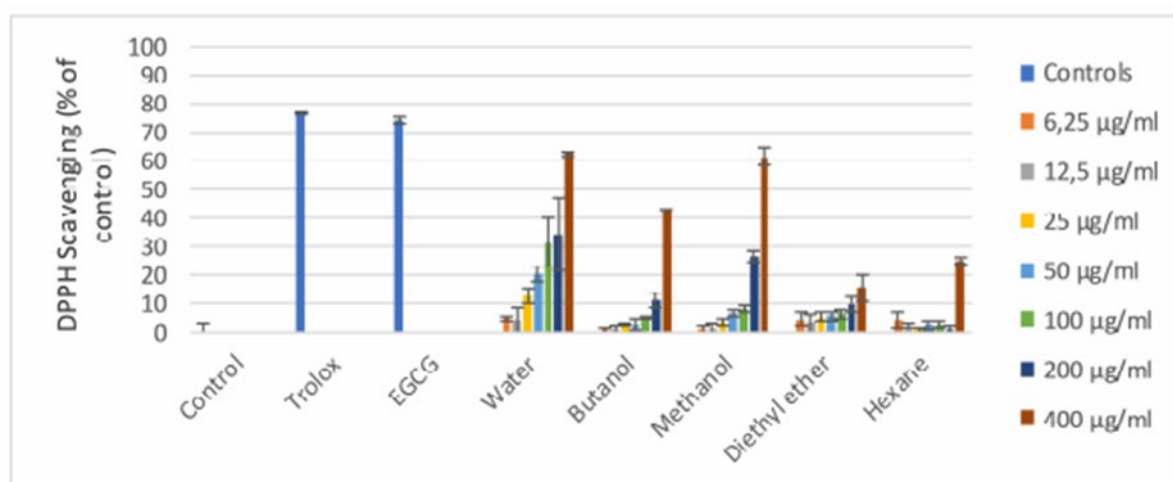


Figure 1. The DPPH scavenging potential of extracts. Trolox and EGCG served as positive controls at 100 µM. Error bars represent standard deviation of quadruplicate values.

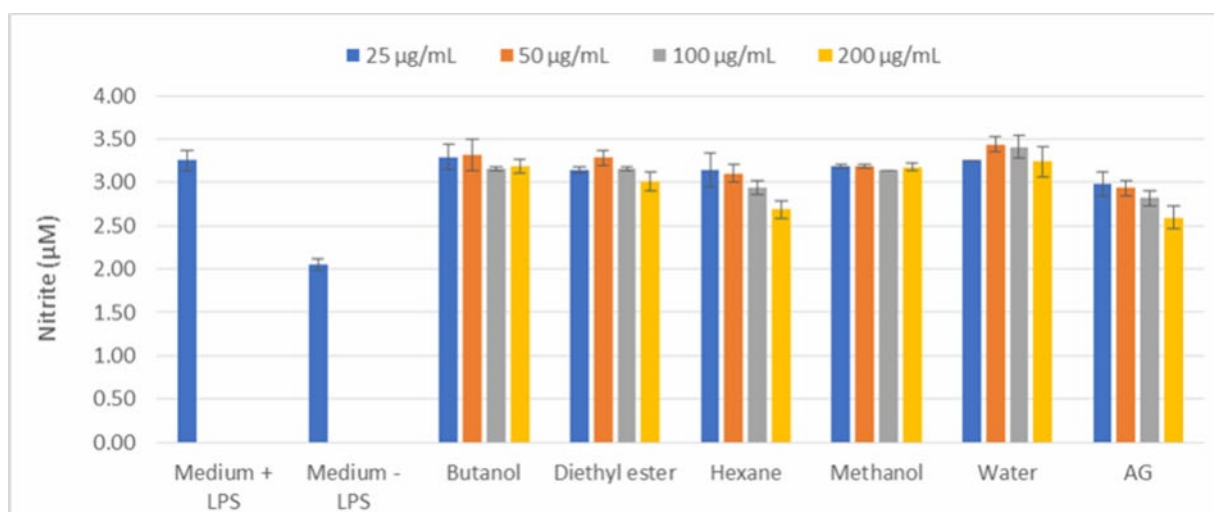


Figure 2. Nitric oxide production in LPS-activated macrophages treated with different concentrations of extracts. Bar graph represents triplicate values of one experiment. Error bars represents the standard deviation of the mean.

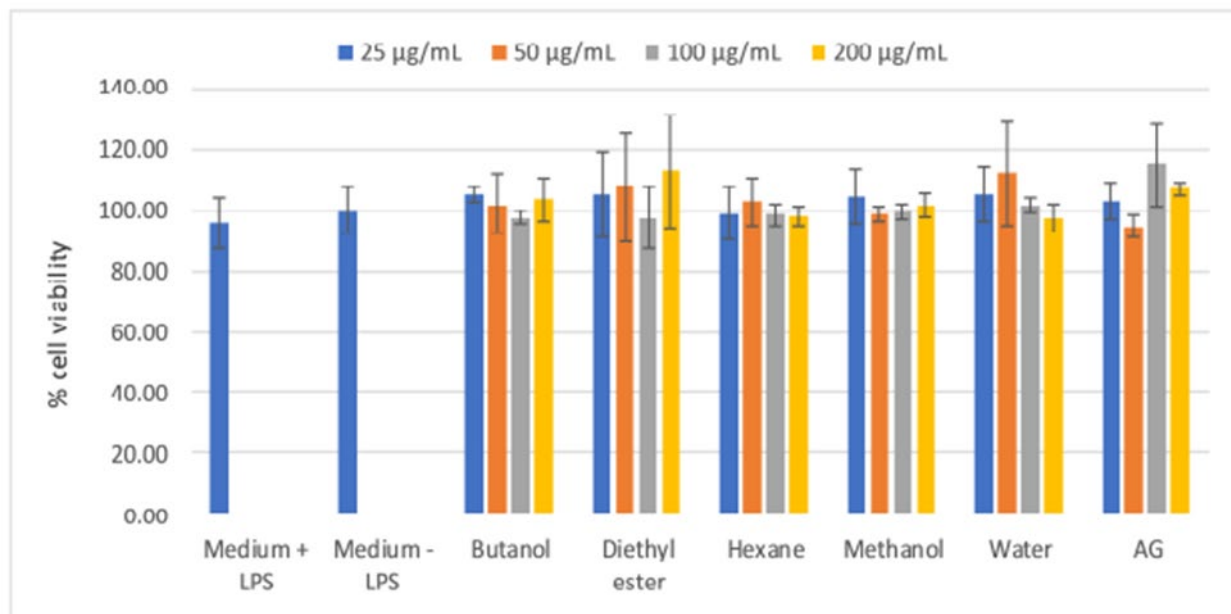


Figure 3. Cell viability (%) of LPS-activated macrophages after 24 hours of exposure to treatments. Bar graph represents triplicate values of one experiment. Error bars represents the standard deviation of the mean.

assay based on electron-transfer that produces a violet solution in ethanol. This free radical, which is stable at room temperature, is reduced in the presence of an antioxidant molecule, thus giving a colourless ethanol solution which is measured at 513 nm using a Biotek® Powerwave XS microtiter plate reader (Winooski, VT, USA).

The results showed that the water extract had the best DPPH scavenging activity which indicates that it has the best antioxidant potential (Figure 1). All the other extracts had relatively poor DPPH scavenging activity, except for the butanol and methanol at 400 µg/ml (Figure 1).

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a vitamin E analog that is indicated for the reduction of oxidative stress and consequent oxidative-stress damage. Trolox plays an essential role in antioxidant bioactivity testing such as in the trolox equivalent antioxidant capacity assay (TEAC) and the ferric reducing ability of plasma (FRAP) antioxidant capacity assay.

The so called flavan-3-ol EGCG is a plant secondary metabolite with potent antioxidant activity that is found in abundant concentrations in green tea, white tea, and black tea [19]. Epigallocatechin gallate (EGCG) exhibits lab-based inhibition of tyrosine-phosphorylation regulated kinase 1A(DYRK1A)

activity which potentiated its use in the treatment of Trisomy 21 individuals (Down Syndrome) [19].

Although inflammation is usually associated with a protective or healing response, many chronic diseases are characterised by persistent inflammation ultimately resulting in tissue dysfunction [20]. Macrophages perform a variety of complex microbicidal functions, including surveillance, chemotaxis, phagocytosis, and destruction of pathogenic micro-organisms [21]. Therefore, the development of therapeutics which can non-specifically augment the innate immune response represents a promising strategy to combat classical and emerging infectious agents.

Macrophages represent a highly heterogeneous group of hematopoietic cells present in almost all tissues including adipose. Depending on the trigger, macrophage responses can be divided into two distinct and mutually exclusive activation programs termed classical and alternative.

Classical activation results in a highly inflammatory phenotype and mainly occurs in response to bacterial products such as Lipopolysaccharide (LPS) and Interferon Gamma (IF-γ). These classically activated macrophages produce a myriad of pro-inflammatory signals which can alter the functionality of its surrounding cells. In addition, these

activated cells produce various highly reactive oxidants including nitric oxide (NO), a product of the catalysis of arginine by the enzyme inducible nitric oxide synthase (iNOS).

Five extracts were tested for anti-inflammatory activity as well as their corresponding toxicity using RAW 264.7 macrophage cell line. Figure 2 shows the result of this experiment. Aminoguanidine was used as a positive control. The only sample to show promising anti-inflammatory activity is the hexane extract (Figure 2).

The mouse macrophage cell line, RAW 264.7, is a well characterised and popular model to investigate the anti-inflammatory potential of test samples. Cells are cultured in multi-well plates and activated by exposure to LPS which induces the expression of iNOS with concomitant nitric oxide formation. Changes in NO production are determined by measuring the levels of nitrate in the culture medium. Simultaneous evaluation of cell viability (MTT assay) is used to confirm the absence of cytotoxicity of the test sample. No samples showed any cytotoxicity (Figure 3).

4. CONCLUSION

The urban lifestyle is fast, and as a result fast processed foods are in greatest demand. This modern approach to nutrition, coupled with environmental (cellular radiation) and industrial toxin exposure increases the prevalence of oxidative stress and chronic inflammation [4]. Due to the established negative implications of oxidative stress and chronic inflammation in human diseases, non-drug alternatives are more preferred due to less to no side effects. The consumption of tea as a health-enhancing beverage predates many modern medical interventions in disease prevention and treatment. Our experiments confirm the existing knowledge that medicinal plants exhibit potent pharmacological bioactivity coupled with less to no side effects and easy access and possible commercial production. In our experiments, *Medicago Sativa* L. clearly exhibited significant antioxidant and anti-inflammatory bioactivity and no samples of *Medicago Sativa* L. showed any cytotoxicity. We therefore recommend further purification and fractionation of *Medicago Sativa* L. bio-active compounds for use in antioxidant and anti-inflammatory drug development.

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CONFLICT OF INTEREST STATEMENT

The authors state that there are no conflicts of interest in this work.

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APPENDIX D: DELTA 1 Manuscript IV

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Original Article

Anti-Neoplastic and Cytotoxicity Potency Measuring of Five *Medicago sativa* L. (Alfalfa) Leaf Extracts Towards Melanoma (UACC62), Breast (MCF7), Prostate (PC3), and Colon (HCT116) Cancer Cells

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ABSTRACT

The National Cancer Registry of the National Department of Health of South Africa confirms that in females, the highest incidence of cancers diagnosed in the year 2019 was breast (10 172 cases), cervical (6 945 cases) and colorectal (1 952 cases) cancers, while in males the most diagnosed cancers were prostate, lung, and colorectal neoplasms. The risk factors that promote cancer development, and metastasis or spreading includes excessive alcohol and narcotic usage, carcinogenic infections (oncoviruses) and genetical predispositions. The global incidence of cancer is much likely to increase because of the increased exposure to risk factors (processed fast foods, industrial toxins in air and water) that promote oxidative stress, low grade chronic inflammation, diabetes mellitus, hyperglycemia, and insulin resistance. Because the ancient healer or doctor relied on plants and other natural resources for therapeutic compounds, we wanted to measure the anti-cancer actions induced by our *Alfac-facah* leaf extracts towards four major cancer cells. The growth inhibitory and cytotoxicity activity that was induced by our extracts was measured using the reliable Sulforhodamine B Assay as per the collaborative research program between the CSIR's Biosciences Pharmacology Group and the NCI. Our *Alfac-facah* leaf test material was extracted using five solvents: Ethanol, Methanol, Diethyl-ether, Acetone, and Water. Medicinal Plants persist to play a key role in medicine, whereby they not only help with treating or preventing diseases, but they also contribute to the general wellbeing of the patient. For this reason, natural resources remain a pivotal ingredient of novel drug development compounds.

Key words: NCI (National Cancer Institute, U.S Department of Health and Human Services), CSIR (The Council for Scientific and Industrial Research, South Africa), *Medicago sativa* L. (Alfalfa), Sulforhodamine B Assay (SRB), ECACC (European Collection of Authenticated Cell Cultures, UK), Cancer (malignancy, neoplasm, carcinoma, tumour, sarcoma, leukemia, lymphoma), AICR (American Institute for Cancer Research), International Agency for Research on Cancer (IARC), THC (*delta-9 tetrahydrocannabinol*). Rick Simpson oil (RSO).

INTRODUCTION

The American antique collector and dealer Edwin Smith, in 1862 procured the ancient medical scroll (the manual for military surgery also known as "the secret book of the Physician") from Egypt, the scroll contained earlier cases of injuries, fractures, wounds, dislocations, and tumours and means to cure/treat them. The authorship of this text is debated but it has been assumed to be written by Imhotep, who was the ancient Egyptian architect that was later (2000 years after his death) venerated as the God of medicine and healing. The ancient medical treatise *Edwin Smith Papyrus* indicates how far back in time humans have been facing the burden of cancer and other medical conditions. The fossilised bone tumours observed on ancient Egyptian mummies also confirms that cancer is an old problem that has now become the second leading cause of death according to the WHO.¹

According to the World Health Organization (WHO) cancer is among the leading causes of death in the world, accounting for 8.2 million deaths in 2012.² International Agency for Research on Cancer (IARC) estimates that more than 60% of

the world's new annual cases occur in Africa, Asia, Central and South America.³ These regions account for approximately 70% of the world's cancer deaths. Even though 30% of cancers can be prevented according to WHO, the IARC estimates that there were 32.6 million people living with cancer in 2012, and the number of new cancer cases is expected to rise from 14 million in 2012 to 22 million cases over the next two decades.³ The current cancer treatment's efficacy is threatened by physical and emotional side effects experienced by hundreds of cancer patients worldwide. Cancer treatment side effects include chemo brain (memory lapse), peripheral neuropathy, pain, fatigue, lymphoedema, infections, infertility and reduced sex drive, nausea, vomiting and many more. This rationalizes the basis for need for rapid development of novel, non-cytotoxic and non-genotoxic anti-cancer agents. Carcinogenesis is documented as a multistep process in which distinct molecular and cellular alterations occur. From the studies of experimentally induced carcinogenesis in rodents, tumour development is considered to consist of several separate, but closely linked, stages: tumour initiation, promotion, and progression.⁴⁻⁶ Blocking agents inhibit carcinogens

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from reaching the target sites, from undergoing metabolic activation or from subsequently interacting with crucial cellular- macromolecules (for example, DNA, RNA, and proteins). While Suppressing agents on the other hand, inhibit the malignant transformation of initiated cells.^{7,8} Chemo-preventive phytochemicals can block or reverse the premalignant stage (initiation and promotion) of the multistep carcinogenesis. They can also halt or at least retard the development and progression of precancerous cells into malignant ones. But plants can also deal with factors that promote oxidative stress, inflammation, and hyperglycemia thereby making conditions uncondusive for malignant cells to grow and spread.⁹⁻¹²

Numerous intracellular signal-transduction pathways converge with the activation of the transcription factors NF- κ B and AP1, which act independently or coordinately to regulate target-gene expression.^{7,8} Atypical activation of NF- κ B has been associated with protection against apoptosis and stimulation of proliferation in malignant cells,^{7,8} and overexpression of NF- κ B is causally linked to the phenotypic changes that are characteristic of neoplastic transformations.⁸ Many chemo preventive phytochemicals that are derived from the diet have been shown to suppress constitutive NF- κ B activation in malignant cells or NF- κ B activation induced by the external tumour promoter phorbol 12-myristate 13-acetate (PMA) or tumour-necrosis factor- α (TNF- α).^{9,13} The conventional approach to cancer treatment is based on drugs that retard the proliferation of abnormal cells (cytostatic) or that can kill the abnormal cells (cytotoxic). The mechanisms of actions employed by these drugs includes the inhibition of cell division or by the killing of neoplastic cells by the induction of apoptosis (suicide by cells). Current anti-cancer drugs like chemotherapy primarily target cell division components, consequently their toxicity is not restricted to cancer cells but healthy cells as well such hair cells, hence alopecia. Many conventional drugs also induce genetic damages that can turn out to be carcinogenic at times. A segment of the drug development research community is focusing on identifying novel chemotherapeutic agents/compounds from plants and other natural resources that do not only induce the untoward side effects of conventional therapeutic chemicals, but that are also cheap and easily accessible or that can be grown at home.^{10,13-16}

The use of plants and their extracts in treatment of diseases dates back before 460-370 BC when Hippocrates practiced the art of healing by the use of plant-based drugs. The medicinal value of plants lies in the contained phytochemical (secondary metabolites) substances that can induce a definite physiological action in the human body. Natural products from natural resources (plants, etc) continue to be used in pharmaceutical preparations either as crude extracts, fractions, pure compounds, or analogous compounds.¹⁷ Traditional medical system of time past still possesses great therapeutic value and also many medicinal plants have been used thus far to synthesize novel therapeutic compounds based on indigenous pharmacopoeias. The most bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolic compounds.¹⁸ Many clinical trials on the use of nutritional supplements and modified diets to prevent cancer are also continuing. It is plausible that in the future people might only need to take specially synthesized pills that contain substances derived from edible plants to prevent cancer or delay its onset. More than 250 population-based studies, including case-control and cohort studies, indicate that people who eat about five servings of fruit and vegetables a day have approximately half the risk of developing cancer — particularly cancers of the digestive and respiratory tracts.^{4,5,19}

The therapeutic potential of merging the traditional/indigenous/ alternative/homeopathic herbal medicine and the modern conventional clinical medicine is evident in Japan where cancer patients now are also prescribed medicines from the *Kampo* traditional pharmacopeia that is said to originate from ancient China. The *Kampo* medical care system is easily accessible through the ancient treatise titled *Shang*

Han Lun by Zhang Zhong Jing, and out of that ancient scroll, one potent anticancer medicine is listed therein as *Juzentaihoto* (JTT) or the so-called *Shi-Quan-Da-Bu-Tang* in Chinese medicine. Shintaro Ishikawa and others, demonstrated the B16 melanoma metastasis inhibition *in-vivo* that was induced by the JTT extract.²⁰ Ishikawa *et al* demonstrated the activity by injecting (intravenously) C57BL/6 male mice with B16 melanoma cells (2×10^5 cells) and feeding them food containing 3.0 % JTT. The mice were then sacrificed after 3 weeks (21 days) following treatment, and tumour cell colonies on the surface of the lung were counted to measure pharmacological potency of JTT. The experimental results demonstrated the therapeutic actions of JTT being to block B16 Melanoma cancer cells: because mice that was not treated with JTT had more than 50 malignant cell colonies, while those that were treated with JTT had less than 20 colonies of metastatic tumour cells.²⁰ This experiment supports the known fact that many plants can help with preventing cancer development and spreading, but they can also help with the effects of cancer treatment. The therapeutic potency of compounds from plants cannot be denied, evidence has been and is still being published on how medicinal plants continue to provide therapeutic metabolites that are antidiabetic, antimicrobial, hepatoprotective, chemo-preventive, antioxidant, anti-inflammatory, and anti-cancer among many others. Docetaxel (DTX) is a semisynthetic analog of Paclitaxel which is isolated from European yew (*Taxus baccata*) that is well used in clinical chemotherapy.²¹ DTX is approved and used in clinical care against breast cancer, ovarian cancer, and non-small lung cancer. DTX was demonstrated to cause cell cycle arrest by interfering with microtubules functions.²¹ The aim of this paper was to examine the anti-cancer potential of *Medicago sativa* L. leaf extracts towards clinical cancer cells. We report here the anti-cancer and cytotoxicity activity of five *Medicago sativa* L. extracts towards melanoma (UACC62), breast (MCF7), prostate (PC3), and colon (HCT116) cancer cells for the first time.

MATERIALS AND METHODS

The Plant material, the extraction and the preparation for *in-vitro* anti-cancer and cytotoxicity activity measuring

The plant materials were collected from a nursery and medicinal plant farm located few degrees west of North of Pretoria (South Africa) with the Batch Number MH 71(10kg). The plant material was cultivated by Zizameleni Farming based in Mamogaleskraal, Northwest Province of South Africa. The test material was cultivated using regenerative natural farming principles and the fertilizers used were all natural and certified organics. The material was air-dried and stored in a cool dry area away from light and heat. The dry, grassy, and pale green leaves were extracted using five solvents: Ethanol, Methanol, Diethyl-ether, Acetone, and water. Sixty (60) grams of powdered plant material was extracted in 1 litre (1000 mL) of every solvent. The extracts were filtered using the Buchner funnel and Whatman no.1 filter paper. The extracts were frozen at -40 °C and freeze-dried for 48 hours at a yield of 9 g of dried extracts. The dried extracts were stored at -4 °C until analysis.

Materials (Cell lines) and maintenance

The human cancer cell lines UACC62, MCF7 and PC3 were acquired from the NCI as part of the framework collaborative research program between CSIR and NCI. HCT116 and WI-38 cell line (normal Human Fetal Lung Fibroblast) were obtained from ECACC (European Collection of Authenticated Cell Cultures, UK). Cell lines were routinely maintained as a monolayer cell culture at 37°C, 5% CO₂, 95% air and 100% relative humidity in RPMI containing 5% fetal bovine serum, 2 mM L-glutamine and 50µg/ml gentamicin. Emetine, Parthenolide, glassware, apparatus and other materials used for our experiments were sourced from reputable supplies that include Merck, United Scientific, Minema, B&M Scientific, and Lasec).

Protocol for the growth inhibitory activity measuring of five *Medicago sativa* L extracts

The SRB Assay was developed by Philip Skehan and others that was chosen for its rapidity, sensitivity, and low cost of materials.²² For the anti-cancer screening experiment, the cells (3-19 passages) were inoculated in a 96-well microtiter plates at plating densities of 7-10 000 cells/well. The plates were then incubated for 24 h. After the 24 h incubation, the cells were treated with the experimental drugs/extracts which were previously dissolved in DMSO and diluted in medium to produce 5 concentrations. Cells without drug addition served as control. The blank contains complete medium without cells. Parthenolide was used as a standard. The plates were incubated for 48 h after the addition of the compounds. Viable cells were fixed to the bottom of each well with cold 50% trichloroacetic acid, washed, dried, and dyed by SRB. Unbound dye was removed, and protein-bound dye was extracted with 10mM Tri's base for optical density determination at the wavelength 540 nm using a multi-well spectrophotometer. Data analysis was performed using GraphPad Prism software. 50% of cell growth inhibition (IC₅₀) was determined by non-linear regression.

Protocol for the cytotoxicity activity measuring of five *Medicago sativa* L extracts

For screening experiment, the cells (21-50 passages) were inoculated in a 96-well microtiter plates at plating densities of 10 000 cells/well and were incubated for 24 h. After 24 h the cells were treated with the experimental drugs which were previously dissolved in DMSO and diluted in medium to produce 5 concentrations. Cells without drug addition served as control. The blank contains complete medium without cells. Emetine was used as a standard. The plates were incubated for 48 h after addition of the compounds. Viable cells were fixed to the bottom of each well with cold 50% trichloroacetic acid, washed, dried, and dyed by SRB. The unbound dye was removed, and protein-bound dye was extracted with 10mM Tri's base for optical density determination at the wavelength 540 nm using a multi-well spectrophotometer. Data analysis was performed using GraphPad Prism software. 50% of cell growth inhibition (IC₅₀) was determined by non-linear regression.

RESULTS

Results are shown in figures 1 to 12.

DISCUSSION AND CONCLUSION

Again, Cancer describes an anthology of diseases of higher multicellular organisms, characterized by mutations in the expression of multiple genes, leading to dysregulation of the normal cellular processes of cell division and cell differentiation.²³ This results in an imbalance of cell replication and cell death that favours growth of a tumour cell population. Cancer cells replicate fast and invade other tissue sites causing significant morbidity and if untreated or diagnosed late, mortality. The cost of living is already high, with consumers struggling to wrestle with the effects of inflation especially now after the COVID-19 pandemic. Despite modern advances in clinical care, the cost of treatments has been reported by the AICR to be costing consumers a staggering US\$895 billion per year. The cost of treating cancer is not only from the hospitals/clinics (diagnosis, drugs, treatment, and admission fees) but also there are non-health care costs related to being a cancer patient. It is a well document fact that many cancer drugs causes for example alopecia and many other physio-psychological side effects that include weight loss and pain/fatigue, factors that can promote mental health issues in those who are living with cancer and their families. There are also costs related to the logistics of going to treatment sessions (chemotherapy, radiotherapy and so on), to this cost you can add mental health support, specialised foods and supplements, bras, or breast prostheses (following mastectomy), wigs (to cover head due to alopecia) and in some cases unpaid leave from work.

The SRB Assay was developed by Philip Skehan and others and was chosen for our experiments for its rapidity, sensitivity, and low cost of materials.²² The method development and design were motivated by the high throughput of experimental needs of the NCI *in-vitro* anti-cancer drug development screening project, which required millions of culture wells per year. SRB is a reliable bright pink amino xanthene dye with two sulfonic groups. The histochemistry of SRB is similar to that of related protein dyes such as bromophenol blue, naphthol yellow and so on. Skehan *et al* compared SRB to other dyes based on its sensitivity, staining intensity and signal-to-noise ratio, and found that SRB was the same to some fluorescent dyes and superior to many other conventional dyes. The other advantages of the SRB assay is that the 100-fold range of linearity far exceeds that of the Lowry and Bradford assays, as thus it eliminates the need for time-consuming dilutions of samples with high protein contents. The SRB Assay also allows a sensitive measuring of drug cytotoxicity in culture. The SRB assay is also comparatively

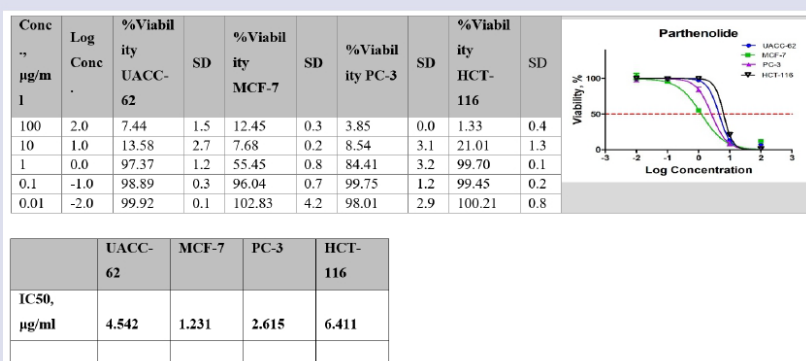


Figure 1: The experimental results of parthenolide (standard) and graphical representation of readings
Z' factor: 0.9

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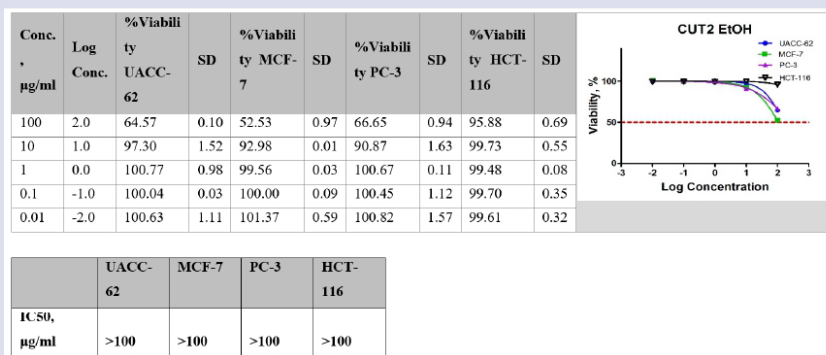


Figure 2: The experimental results of the ethanol extract (CUT2 EtOH) and graphical representation of readings
Z' factor: 0.9

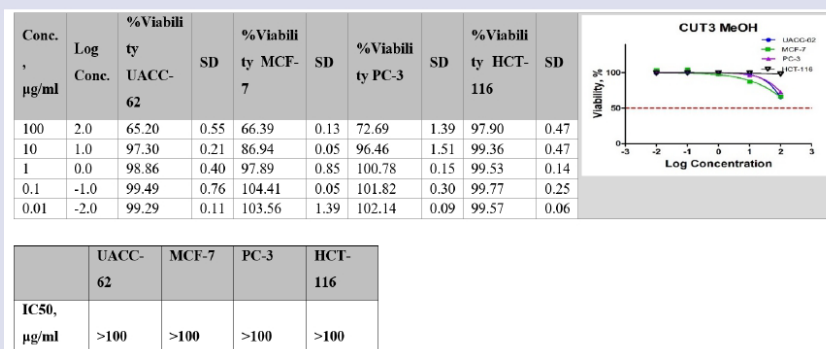


Figure 3: The experimental results of the methanol extract (CUT3 MeOH) and graphical representation of readings
Z' factor: 0.9

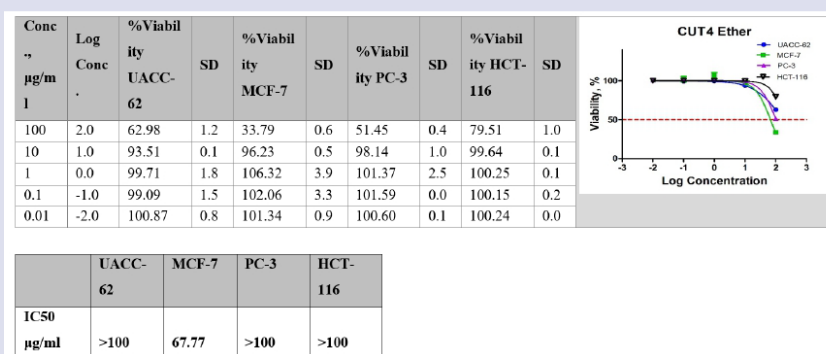


Figure 4: The experimental results of the diethyl-ether extract (CUT4 Diethyl-Ether) and graphical representation of readings
Z' factor: 0.9

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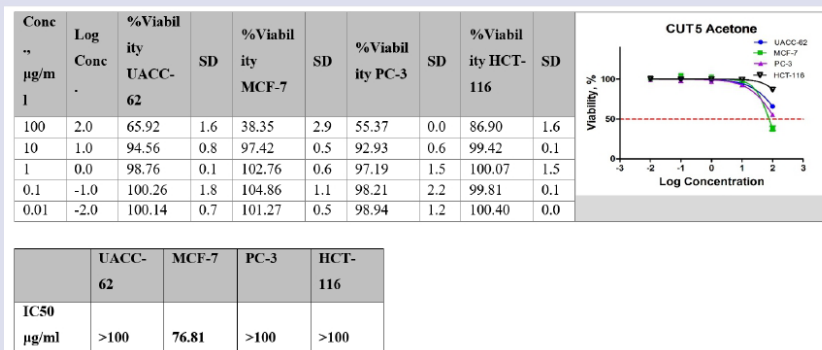


Figure 5: The experimental results of the acetone extract (CUT5 Acetone) and graphical representation of readings
Z' factor: 0.9

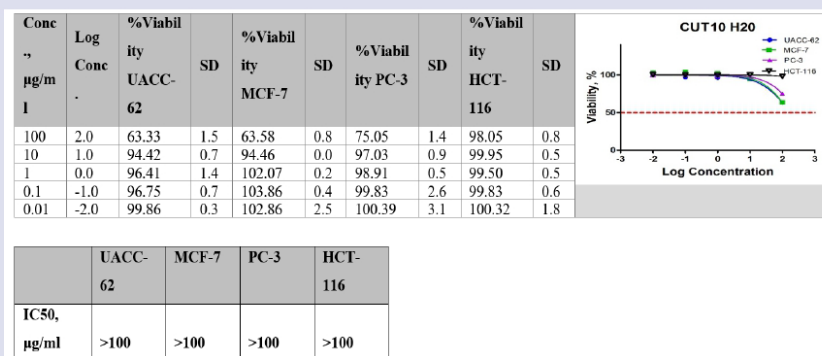


Figure 6: The experimental results of the acetone extract (CUT10 H2O) and graphical representation of readings
Z' factor: 0.9

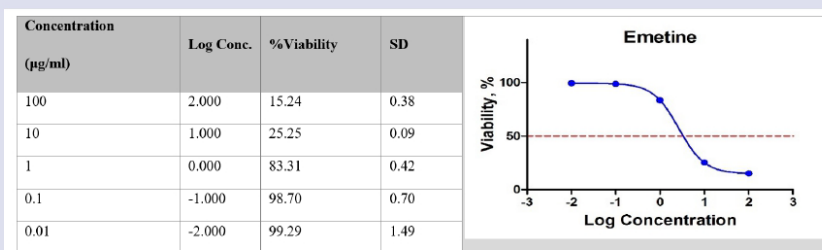


Figure 7: The experimental results of the standard (Emetine) and graphical representation of readings
Z' factor: 0.9, IC50: 2.66 μg/ml

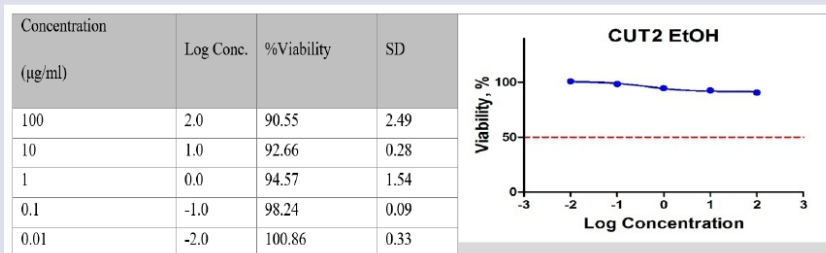


Figure 8: The experimental results of the ethanol extract (CUT2 EtOH) and graphical representation of readings
Z' factor: 0.9, IC50: >100 $\mu\text{g/ml}$

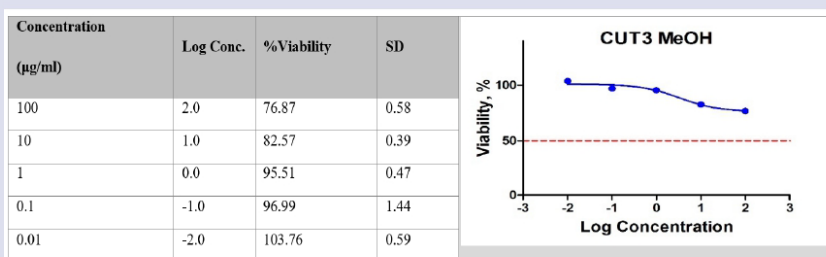


Figure 9: The experimental results of the methanol extract (CUT3 MeOH) and graphical representation of readings
Z' factor: 0.9, IC50: >100 $\mu\text{g/ml}$

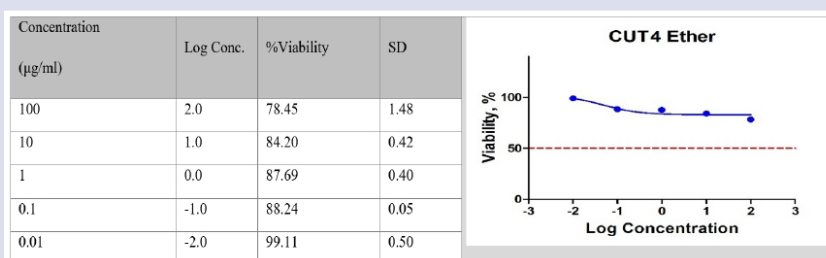


Figure 10: The experimental results of the diethyl-ether extract (CUT4 Diethyl-Ether) and graphical representation of readings
Z' factor: 0.9, IC50: >100 $\mu\text{g/ml}$

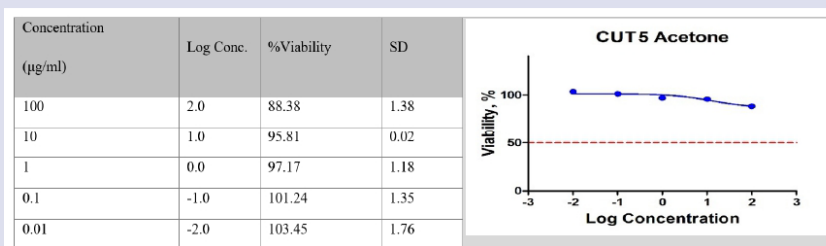


Figure 11: The experimental results of the acetone extract (CUT5 Acetone) and graphical representation of readings
Z' factor: 0.9, IC50: >100 $\mu\text{g/ml}$

Simon MT, et al. Anti-Neoplastic and Cytotoxicity Potency Measuring of Five *Medicago sativa* L. (Alfalfa) Leaf Extracts Towards Melanoma (UACC62), Breast (MCF7), Prostate (PC3), and Colon (HCT116) Cancer Cells

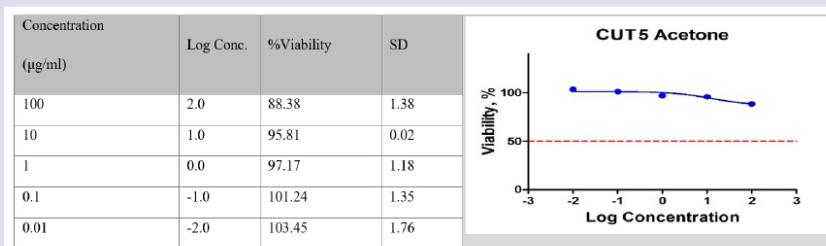


Figure 11: The experimental results of the acetone extract (CUT5 Acetone) and graphical representation of readings
Z' factor: 0.9, IC50: >100 µg/ml

Table 1: The summary of inhibitory effects of alfalfa extracts and the standard towards four cancer cells.

No. Sample	IC50 for UACC-62, µg/ml	IC50 for MCF-7, µg/ml	IC50 for PC-3 µg/ml	IC50 for HCT-116 µg/ml
1 CUT2 EtOH	>100	>100	>100	>100
2 CUT3 MeOH	>100	>100	>100	>100
3 CUT4 Ether	>100	67.77	>100	>100
4 CUT5 Acetone	>100	76.81	>100	>100
5 CUT10 H2O	>100	>100	>100	>100
Parthenolide	4.542	1.231	2.615	6.411

Table 2: The summary of cytotoxicity measuring of alfalfa extracts and the standard towards four cancer cells.

No.	Name of sample	IC50 (µg/ml)
1	CUT2 EtOH	>100
2	CUT3 MeOH	>100
3	CUT4 Ether	>100
4	CUT5 Acetone	>100
5	CUT10 H2O	>100
	Emetine	2.66

simpler, faster, and more sensitive than the MTT assay.²² We employed the SRB assay to measure the anti-cancer potency of five Alfalfa extracts at five different concentrations.

For our experiments we used plant-derived standards that are super potent: Parthenolide (PH) and Emetine. Parthenolide is a sesquiterpene lactone that occurs naturally in the leaves, flowers, and fruits of the feverfew plant (*Tanacetum parthenium*). PH has been demonstrated to inhibit nuclear factor kappa B signaling, induction of apoptosis and the reduction of subpopulation of cancer stem-like cells in various cancers. For our experiment, the plant material was extracted using five solvents: Ethanol (CUT2 EtOH), Methanol (CUT3 MeOH), Diethyl-ether (CUT4 ether), Acetone (CUT5 Acetone), and Water (CUT10 H2O). following treatments, optical density (OD) was determined at the wavelength of 540 nm using a multi-well spectrophotometer. Data analysis was performed using GraphPad Prism software. 50% of cell growth inhibition (IC50) was determined by non-linear regression. The readings and graphical readings of tests are recorded here as 3.1 to 3.12. for measuring pf anticancer and cytotoxicity actions we worked with samples/standard of 100 – 0.01 µg/ml (5 x 10-fold serial dilutions) concentration. The summary of the results of our anti-cancer potency testing are summarized in Table 1. According to criterion of the CSIR the sample is considered Weak if parameter IC50 for two or three cell lines is more than 15 µg/ml but less than 100µg/mL. Therefore, all tested samples can be said to be inactive (3.1 – 3.6). The diethyl-

ether and acetone extracts displayed some weak activity towards the MCF-7 breast cancer cell line (3.4 and 3.5) with the 50% inhibitory concentration of 67.7 µg/mL and 76.81 µg/mL respectively.

Emetine is a pyridoisoquinoline that is used clinically as an emetic, antiprotozoal, antiviral, antimalarial, and antineoplastic agent. Emetine is a natural alkaloid that is found in European Ivy (*Hedera helix*), *Alagium longiflorum* and others. Emetine is used clinical an amoebicidal drug, but it also possesses antineoplastic properties, where it induces apoptosis but also suppress the viability, migration, invasion, and sphere formation of breast cancer cells. The cytotoxic effects of the compounds were tested by Sulforhodamine B (SRB) assay on the WI38 cell line (normal Human Fetal Lung Fibroblast from ECACC). For our testing we worked with samples/standard of 100 – 0.01 µg/ml (5 x 10-fold serial dilutions) concentration. The summary of the results of our cytotoxicity potency testing are summarized in Table 2.

The CSIR criteria considers samples with IC50 of more than 100 µg/ml a low hazard/low potency, while samples that have IC50 of less than 5 µg/ml are regarded as high hazard / potent. The cytotoxicity dose dependant curves (3.7-3.12) clearly show the cytotoxicity potency of our standard: Emetine, and the experimental results confirms that none of our extracts exhibited notable bioactivity v (3.7-3.12). *Medicago sativa* L is a well-known plant around the world, with common names that includes Alfalfa, Lucerne, Buffalo Herb, Purple Medic/Medick/Medicle and many others.¹⁵ Alfalfa is mostly used in agriculture as a fodder crop, where it provides beneficial nourishment to animals and humans. The herbaceous lucerne plant is indeed a multi-purpose crop, that benefits the farmer who grows it, the livestock rancher who uses it to feed animals during times of drought or winter, and for the human being who vows by the health and wellness enhancement (vitality) potency of alfalfa sprouts and tea from leaves and as well as part of commercial herbal concoctions. Lucerne is very high in proteins, vitamins, and phytoestrogens. The Purple Medic plant actually has environmental benefits as well, it has been used to prevent soil erosion for example by binding the soil particles together. Although the plant is safe to be consumed by animals and humans, we found in literature that it also possessed some risk, wherein the seeds are said to contain L-canavanine, an amino acid that is detrimental to patients with the autoimmune disease called lupus. Otherwise *Medicago sativa* has been regarded to be beneficial for health and wellness, where many experimental and ethnobotanical reports demonstrated therapeutic effects towards diabetes (lowering hyperglycemia), lowering cholesterol, acting as a diuretic, and to increase lactation and to promote menstruation just to mention a few. In the year 2003, Rick Simpson a Canadian Engineer was diagnosed with a form of skin cancer called basal cell carcinoma. As a general enthusiast of cultivating the age-old controversial medicinal plant of our ancestors: *Cannabis sativa* (called by many names: *matekoane*, weed, holy herb, *ganja*, *hemp*, *marijuana*, *Marijuana*, etc),

acting from hearsay evidence that marijuana is potent towards cancer, Rick decided to apply the oil he extracted from *matekoane* (*Cannabis sativa*) on the affected area and covering it with a bandage. Mr Simpson claims these actions were motivated by the scientific validation study he read from the National Cancer Institute (NCI) trial where THC (*delta-9 tetrahydrocannabinol*) was demonstrated to kill cancer in mice. Several days following the application of the oil, Rick removed the bandage to find the cancer gone and the area was healed. As a result of this “discovery”, Rick started to produce the full cannabis extract that is now referred to Rick Simpson oil (RSO), but because it still contained the psychoactive ingredient THC, and it was (and still is) illegal to sell or buy in many parts of the world. The risk factors that promote cancer in humans have increased due to industrialization and the commercial use of toxic and carcinogenic compounds in food, toothpaste, water, air and agriculture. The safety of radiofrequency waves such as 5th Generation Mobile Network (5G) implications on the human body, although the WHO assures that there are no envisioned health risks from 5G as consequent tissue heating caused by the exposure is below international guidelines of electromagnetic fields exposure (The International Commission on Non-ionizing Radiation Protection and The International Committee on Electromagnetic Safety). The burden of diseases such as cancer, diabetes, hypertension, and mental health disorders is evident everywhere we live, it is the moral obligation of the devotees of science to devise the needed solutions. It was for this rationale that we found it necessary to measure the anti-neoplastic actions induced by the world-revered *Medicago sativa* and our experimental results as a whole clearly validate the ethnobotanical views that alfa-alfa has medicinal benefits to humans, animals, and the environment. We therefore recommend further scientific testing of the Purple Medic, and we rely on our experimental results published here and elsewhere that the religious drinking of alfalfa tea can be beneficial for health and wellness. Even though our extracts did not induce comparable potency of other compounds or plants, we still we recommend the combination of Alfalfa and *Sutherlandia frutescens* (otherwise known as cancer bush, *kankerbos*) for a potent non-drug-based supplement for health and wellness particularly towards the prevention of cancer, diabetes, inflammation, and oxidative stress. And for those with mental health issues we recommend the addition of *gingko biloba*.

CONFLICTS OF INTEREST

The authors declare no conflicts of interests regarding the publication of this paper. The views expressed in this text are of the Authors only and does not reflect the views of CUT.

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APPENDIX E: CONFERENCE INVITES / ABSTRACT ACCEPTANCE



International E-Conference on **Traditional Medicine** April 14-15, 2022 | Webinar

ABSTRACT ACCEPTANCE LETTER

To
Dr. Thapelo Simon Makhele,
Central University of Technology,
Free State Province, South Africa.

February 11, 2022.

United Research Forum, UK cordially invites **Dr. Thapelo Simon Makhele** to attend the **International E- Conference on Traditional Medicine** scheduled during **April 14-15, 2022**.

On behalf of the Organizing committee, we welcome you to join with your Poster Presentation and share your knowledge on the theme *"Nature itself is the best Physician"*

We are glad to inform you that your abstract, entitled

THE IN-VITRO GLUCOSE-UTILIZATION ACTIVITY TESTING OF FIVE Medicago sativa L. (Alfalfa) LEAF EXTRACTS TOWARDS HEPG2/C3A AND L6 MUROID CELL LINES.

Has been accepted for the **Poster Presentation** at *Traditional Medicine Webinar 2022*. In this regard your abstract was peer reviewed and the abstracts would be published in conference book/proceedings.

We look forward to seeing you on Screen April 14-15, 2022.

For more details about Traditional Medicine Webinar 2022
<https://unitedresearchforum.com/traditionalmedicine-conference/>

With our best wishes,
Emma Watson
Program Director | ICTM- 2022
Phone: +44 7915608502
Email: emmawatson@scientificmeetings.co.uk



This message is from an external address

Dear **Mr Thapelo Simon Makhele**

Thank you for your interest in participating at the ATNPM2022 Conference. Your Poster review status is :
Accept

Official invitation to attend the ATNPM 2022 conference

Thank you for your submission to the African Traditional and Natural Product Medicine Conference to be held from 19 to 21 October 2022 at The Ranch Protea Hotel, Polokwane, South Africa.

We are pleased to inform you that your abstract submission has been accepted for presentation after scientific review.

If necessary, you may use this letter for funding or visa purposes. Alternatively, an official acceptance letter will be sent for logistical arrangement upon request.

The programme for the conference will be finalised early in September and will be posted on the conference website (www.ul.ac.za/atnpm2022).

For more information about the conference, please visit our website by clicking the attached link: (www.ul.ac.za/atnpm2022).

We are looking forward to hosting you at the upcoming conference.

Kind Regards

ATNPM Scientific Committee



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Athens, 30 March 2023

Thapelo Simon Makhele, Technical Assistant
Pakiso Moses Makhoahle, Professor
Sitheni Samson Mashele, Professor
Central University of Technology
South Africa

Our Ref: HSC2023/1304021

Dear Colleagues,

I would like to inform you that the selection academic committee has decided to invite you to speak at our 11th Annual International Conference on Health & Medical Sciences 1-4 May 2023 Athens, Greece, on the topic of:

The In-Vitro Glucose-Utilization Activity Testing Of Medicago Sativa L. (Alfalfa) Leaf Extracts In L6 and Hepg2/C3a Cells

Please be prepared for an oral presentation in English only of 15-20 minutes including discussion. During your presentation you may use PowerPoint. If for objective or subjective reasons because of the new ecumenical situation of pandemics an onsite presentation is not possible, ATINER will accept and facilitate remote (online or pre-recorded) presentations. If this is your first presentation at one of ATINER's small academic events, please read carefully our policy available at <https://www.atiner.gr/policy>.

More information can be found at the conference website <https://www.atiner.gr/healthsciences>. Confirm your participation by completing the registration before 10 April 2023 at: <https://www.atiner.gr/product/registration>.

If you want to book your hotel accommodation with ATINER, please fill the form at <https://www.atiner.gr/hotel-reservation> and our administration will respond to your request asap. Reservations should be made as soon as possible as the offer only stands as long as rooms are available. If you need more information on the accommodation, please send us an email to atiner@atiner.gr.

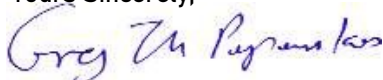
The conference's social program is available on <https://www.atiner.gr/social-program>. These are academic events which give the opportunity to our conference participants to further discuss the issues developed during the formal sessions and establish academic collaborations with other scholars from many different countries.

Please note that participants have the option to pay in Canadian or US Dollars through bank transfer in a North American Bank. If you prefer this option, please send us an email and we will send bank accounts and rates in the two currencies.

If you want your paper to be considered (peer reviewed) for publication and only then, please submit your manuscript by 1 May 2023 using our website <https://www.athensjournals.gr/paper-submission> following the paper guidelines stated there. For more information, please see point 3 of our policy on <https://www.atiner.gr/policy>. Please do not submit your paper if you do not want it to be published by ATINER.

I look forward to meeting you in Athens.

Yours Sincerely,



Dr. Gregory T. Papanikos
President

