University of KwaZulu-Natal

College of Health Sciences, School of Clinical Medicine

Identifying mechanisms associated with metronidazole resistance in *Trichomonas vaginalis* and investigating newer therapeutics against this pathogen

Presented by

Rowen Govender

215023500

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy (Medicine)

in the School of Clinical Medicine,

University of KwaZulu-Natal

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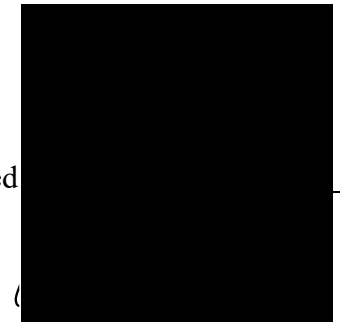
Signed  Date: 10 November 2023

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DECLARATION BY SUPERVISOR

As the candidate's supervisor, I Prof Nathlee Abbai agree to the submission of this thesis.

Signed:  Date: 13 November 2023

RESEARCH OUTPUTS

Accepted Manuscript

1. **Rowen Govender**, Nonkululeko Mabaso and Nathlee S Abbai. Investigating the Associations between *Trichomonas vaginalis* and its endosymbionts (*T. vaginalis virus* and *Mycoplasma hominis*) in relation to metronidazole resistance. Manuscript accepted by the Journal of Infection in Developing Countries (JIDC 17592)

Submitted Manuscripts

1. **Rowen Govender** and Nathlee S Abbai. The role of iron on the growth and metronidazole activity in *Trichomonas vaginalis*. The manuscript is under review in Parasitology (PAR-2023-0096).
2. **Rowen Govender**, Deshanta Naicker, Swenkile Ndlovu and Nathlee S Abbai. Synergistic Strategies: Harnessing Plant Nanoemulsions to Combat Metronidazole-Resistant *Trichomonas vaginalis* in South Africa. This manuscript is under review in Infectious Diseases in Obstetrics and Gynecology (ID 1962038)
3. **Rowen Govender**, Swenkile Ndlovu and Nathlee S Abbai Nanocomplex-Based siRNA Delivery: Expanding Frontiers in *Trichomonas vaginalis* Infection Control and Precision Medicine. This manuscript is under review in Parasitology Research (Submission ID: 88a878e9-3f41-46a7-a8bb-a6c17c41c591)

Conference Presentations

1. **Rowen Govender,** Nonkululeko Mabaso and Nathlee S Abbai. Investigating the Associations between *Trichomonas vaginalis* and its endosymbionts (*T. vaginalis virus* and *Mycoplasma hominis*) in relation to metronidazole resistance. Federation of infectious diseases in societies of Southern Africa.

Other manuscripts emanating from this study

1. Deshanta Naicker, **Rowen Govender** and Nathlee S Abbai. Antimicrobial Activity of Plant-Infused Nanoemulsions against *Neisseria gonorrhoeae*. This manuscript is under review in the International Journal of Microbiology (ID 7084347)

DEDICATION

To my Parents and Grandparents, this thesis is dedicated to you, for your unwavering support, love, and guidance throughout my academic journey. Your belief in me has been my greatest motivation, and I am deeply grateful for the values, wisdom, and encouragement you've shared. This achievement is as much yours as it is mine. Thank you for being my pillars of strength.

ACKNOWLEDGEMENTS

In the profound words of Hinduism, "Matha, Pitha, Guru, Daivam," an ancient and revered Sanskrit mantra that translates to 'Mother, Father, Teacher, God,' encapsulates the guiding principles behind the acknowledgments that follow. It is with deep gratitude that I acknowledge the critical roles these four entities played in my life and my academic journey.

- To my parents and grandparents, who are my living Gods, I owe an immeasurable debt of gratitude. Their sacrifices, unwavering love, and guidance have been my guiding lights throughout this academic journey. Their wisdom and support have provided me with the strength to persevere through the countless challenges that come with pursuing a Ph.D. Their values and teachings are the foundation upon which I have built my academic and personal life, and I am forever grateful for their profound influence.
- My exceptional supervisor, Prof. Nathlee Abbai, deserves special mention for her instrumental role in my academic development. Her unwavering guidance, encouragement, and mentorship have been the driving force behind my success in this doctoral pursuit. Prof. Abbai's expertise and profound insights have not only enriched my research but have also deeply impacted my approach to academia. Her support and wisdom have made a lasting imprint on my academic journey.
- I extend my sincere appreciation to my colleagues in the clinical medicine laboratory at the University of KwaZulu-Natal. Our collaborative and enriching academic environment has been pivotal in shaping my research and broadening my perspective. The camaraderie and shared experiences with this talented group of individuals have added a unique dimension to my academic journey.
- To my dear friends, I owe a debt of gratitude that words cannot fully express. Your unwavering support, understanding, and enduring friendship have been the pillars that held me up during

challenging times and the source of celebration during successes. Your presence in my life has been a constant reminder of the importance of balance and camaraderie on this academic path.

- To all those who have played a role in my academic journey, whether through advice, assistance, or simply being a part of my life during this period, your contributions have not gone unnoticed. Your influence, in various ways, has enriched my experience and growth as a researcher. I extend my heartfelt thanks to all who have been a part of this journey.

Funding

- I would like to express my gratitude and thanks to the National Research Foundation (NRF) and the University of KwaZulu-Natal College of Health Sciences (CHS) Scholarship for their generous assistance, which has been instrumental in facilitating my academic endeavours.

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ACRONYMS

AMR	Antimicrobial Resistance
ATP	Adenosine Triphosphate
BREC	Biomedical Research Ethic Committee
BSNs	Biologically Synthesized Nanoparticles
cDNA	Complementary DNA
CDC	Centers for Disease Control and Prevention
CO_2	Carbon Dioxide
Ct	Cycle Threshold
DNA	Deoxyribonucleic Acid
dsDNA	Double-Stranded DNA
dsDNase	Double-stranded Deoxyribonuclease
dsRNA	Double-Stranded RNA
EDTA	Ethylenediaminetetraacetic Acid
EDS	Energy-Dispersive X-ray Spectroscopy
FeNPs	Iron Nanoparticles
HIV/AIDS	Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome
Kbp	Kilo-base pair
MIC	Minimum Inhibitory Concentration
MTT	Methylthiazol Tetrazolium
MTZ	Metronidazole
NAAT	Nucleic Acid Amplification Tests
ng/µl	Nanograms per Microliter

N:P ratio	Nitrogen to DNA Phosphate Ratio
NTR	Nitroreductases
O_2	Oxygen
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
P270	Immunogenic protein
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy
siRNA	Small Interfering RNA
STD	Sexually Transmitted Disease
STI	Sexually Transmitted Infection
TEM	Transmission Electron Microscopy
TE buffer	Tris-EDTA buffer
TVVs	*Trichomonas vaginalis* viruses
TYM	Trichomonas Diamond's TYM Media
UDG	Uracil-DNA Glycosylase
UV	Ultraviolet
WHO	World Health Organization

ABSTRACT

Introduction

Trichomonas vaginalis, the etiological agent of trichomoniasis, a prevalent non-viral sexually transmitted infection, presents a growing public health concern due to the emergence of metronidazole resistance, which compromises the effectiveness of this frontline treatment. To address this challenge, this PhD thesis comprises four research manuscripts aimed at understanding the mechanisms associated with metronidazole resistance in *T. vaginalis* and investigating innovative therapeutic approaches against this pathogen. *T. vaginalis*, a flagellated protozoan parasite, has long been susceptible to metronidazole. However, the alarming increase in drug-resistant strains necessitates a comprehensive examination of this resistance. This study explored the interaction of *T. vaginalis* with endosymbionts, particularly *T. vaginalis* viruses (TVVs) and *Mycoplasma hominis*, delving into their association with drug resistance. Additionally, alternative treatments such as plant-based nanoemulsions and nanotechnology-mediated approaches were investigated. This thesis serves as a beacon of knowledge and innovation in the ongoing battle against metronidazole-resistant *T. vaginalis* isolates, offering new insights and promising strategies for the future of trichomoniasis management.

Methods

This research encompassed a multifaceted methodological approach, systematically examining metronidazole resistance in *T. vaginalis* and exploring alternative therapeutic avenues. The initial set of investigations utilized a combination of *in vitro* studies and molecular assays to analyse clinical isolates of *T. vaginalis*. This entailed scrutinizing the presence of *T. vaginalis* viruses and intracellular *M. hominis* within these isolates and delving into the intricate associations between these endosymbionts and metronidazole resistance. Further studies conducted *in vitro* susceptibility assays, gene expression analyses, and investigated the influence of iron supplementation on metronidazole

resistance in *T. vaginalis* isolates. This multifaceted approach aimed to uncover the role of iron in regulating genes associated with metronidazole resistance and to assess how alterations in iron levels might affect the susceptibility of *T. vaginalis* isolates to the drug. In separate investigations, plant-based nanoemulsions were meticulously prepared from select medicinal plants and rigorously tested for efficacy against metronidazole-resistant *T. vaginalis* isolates, exploring their potential as alternative therapeutic agents. Additionally, our research ventured into the realm of nanotechnology, focusing on the synthesis of iron nanoparticles, modified with chitosan and small interfering RNA (siRNA). This novel approach aimed to explore the potential of these nanocomplexes for gene silencing and targeted therapy in the battle against *T. vaginalis* infections.

Results

The research findings have unveiled valuable insights into the mechanisms associated with metronidazole resistance in *T. vaginalis*. Twenty-one clinical isolates of *T. vaginalis* were included in the endosymbiosis analysis. The prevalence of TVV and *M. hominis* were 76% (16/21) and 86% (18/21), respectively. The presence of any TVV was significantly associated with metronidazole susceptibility patterns (p=0.012). No significant associations were noted between the coinfection of both endosymbionts and metronidazole resistance.

This study went on to show that iron concentrations of 30 µM and 60 µM had led to a loss of resistance in certain isolates of *T. vaginalis*. Furthermore, gene expression analysis indicated that iron played a role in modulating the expression of resistance-associated genes. Additionally, the antimicrobial properties of iron on *T. vaginalis* growth was also evident, which potentially influenced the significance of these gene expression alterations. The efficacy of plant-based nanoemulsions from medicinal plants against metronidazole-resistant *T. vaginalis* isolates was highlighted. The nanoemulsions shifted resistant isolates towards susceptibility in a concentration-dependent manner, with minimum inhibitory concentrations (MICs) decreasing from 4 µg/ml to 1 µg/ml, promising the

restoration of plant-based treatments' effectiveness. Finally, the synthesis of iron nanoparticles modified with chitosan and small interfering RNA (siRNA) for potential gene silencing and targeted therapy against *T. vaginalis* was presented. These nanoparticles were successfully synthesized and efficiently bound with siRNA, offering a potential for innovative therapeutic interventions. Concentration-dependent effects on cell viability were observed, highlighting the need for precise optimization in therapeutic strategies. These comprehensive results collectively signify alternative strategies for addressing metronidazole resistance and mark a significant step in advancing precision medicine and targeted therapies for *T. vaginalis* infections.

Conclusion

The collective findings from this research provides compelling evidence for alternative approaches to address metronidazole resistance in *T. vaginalis*. The multifaceted nature of this thesis illustrates the complexity of metronidazole resistance and the potential for innovative therapies. These discoveries pave the way for a more comprehensive understanding of *T. vaginalis* infections and resistance mechanisms. They hold the promise of precision medicine, drug delivery, gene silencing, and targeted therapies. However, for these research outcomes to translate into effective clinical therapies, further exploration, *in vivo* validation, and rigorous clinical studies are essential. These steps are crucial for addressing the growing challenges posed by *T. vaginalis* infections and metronidazole resistance while ensuring the continued efficacy of treatment options in the field of public health.

IQOQA

Isingeniso:

ITrichomonas vaginalis (TV), i-ejenti eyisisusa setrichomoniasis, isifo esithathelwana ngocansi esingafaki amagciwane, kuveza ukukhathazeka ngempilo yomphakathi ngenxa yokuqubuka kokungezweli kwemetronidazole, okulimaza ukusebenza ngendlela kokwelapha okuhamba phambili. Ukubhekana nale nselelo, le thisisi ye-PhD iqukethe imibhalomicubungulo yocwaningo emine okuhloswe ngayo ukuqonda izindlela ezihlobene nokungezweli kwemetronidazole kwiTrichomonas vaginalis nokuphenya izindlelasu zengxubekwelapha, i-TV, isimuncigazi seflagellated protozoan, okusengcupheni yemetronidazole. Kodwa, ukwenyuka okwethusayo kohlobo olungezweli emithini yokwelapha kuphoqa uhlolo olunzulu lokungezweli. Ucwaningo lubheka ukusebenzisana ne-TV nge-endosymbionts, ikakhulukazi amagciwane eTrichomonas vaginalis viruses (TVVs) neMycoplasma hominis, kubhekwa umthelela wokungezweli. Ukongeza, ukwelashwa okwehlukile njengenanoemulsions kanye nezindlelasu ezingenelela inanotechnology ziyaphenywa. Le thisisi iwuphaphe lwegwalagwala nomzamo empini eqhubekayo elwisana nokungezweli kwemetronidazole ezinhlotsheni ze-TV, okunika imibono emisha nemiqondosu ngekusasa lokulawula itrichomoniasis.

Izindlelakwenza ucwaningo:

Lolu cwaningo luqalise indlelasu yokwenza ucwaningo olungxenyeningi, nokuhlola ngokohlelo lokungezweli imetronidazole kwiTrichomonas vaginalis nokuhlaziya izindlela zokwelapha. Isethi yokuqala yophenyo isebenzise ingxube ocwaningweni lwe-in vitro nama-asayi amamolekhyuli ukuhlaziya iTrichomonas vaginalis. Lokhu kufake ukucwaninga ubukhona bamagciwane eTrichomonas vaginalis viruses (TVVs) neMycoplasma hominis kulama ayisolethi nokungena ekuxhumaneni phakathi kokungezweli kwe- endosymbionts nemetronidazole. Ucwaningo olunye olwenziwe kwi-in vitro engathola ama-asayi, ukuhlaziya ukuchazwa kolibofuzo, nokuphenya umthelela wokuchitshiyelwa kwe-ayoni ekungezwelini kwemetronidazole kwiTrichomonas vaginalis isolates. Le ndlelasu exubile ihlose ukwembula indima ye-ayoni ekusebenzeni ulibofuzo oluhlobene nokungezweli kwemetronidazole nokuhlola ukuba nokuseceleni emazingeni e-ayoni okungaba nomthelela ekubeni khona kweTrichomonas vaginalis emuthini wokwelapha. Ophenyweni olwehlukile, amananoemulsions agxiliswe ezitshalweni alungiswa ngendlela ezitshalweni zokwelapha ezikhethiwe nezihlolwe ngendlela ukubheka ukuthi zisebenza kanjani uma kubhekwa

ukungezweli kwemetronidazole kwiTrichomonas vaginalis, kuphenywa amandla ayo njengama-ejenti engxubekwelapha eseceleni. Ukongeza, ucwaningo olwenziwe endaweni yenanotechnology, lugxile ekugayekeni kwezinhlayiyana ze-ayoni, okwenziwe kabusha ngechitosan ne-RNA encane engenelelayo, ismall interfering RNA (siRNA). Le ndlelasu entsha kuhloswe ngayo ukuphenya ukukwazi kwala mananocomplexes ukuthulisa ulibofuzo nengxubekwelapha ehlosiwe empini yokulwisana nokutheleleka ngeTrichomonas vaginalis.

Imiphumela:

Imiphumela yocwaningo iveze imibono enomqondo onzulu ngezindlela ezihlobene nokungezweli kwemetronidazole kwiTrichomonas vaginalis. Ukuvama okukhulu kwamagciwane eTrichomonas vaginalis (TVVs) kwenziwa ngama-76% wama-ayisoleythi okwelapha, neMycoplasma hominis esezinhlayiyeni ezingxenyeni ezingama-86%. Okuqaphelekayo, ukuthi ubukhona be-TVVs abuvezanga ukuhlobana okubalulekile kwezimpawu zokugula noma ukungezweli kwemetronidazole, ukukhanyisa ukunikezelana okunkimbi kwalezi zilwanyana nesimuncigazi. Nakuba ucwaningo luveze ukuthi umthamokumumatha weTrichomonas vaginalis' ukusungula ubudlelwane be-endosymbiotic ngezilwanyana eziningi eziphilayo, lezi zinhlangano azibanga nomthelela omkhulu ekungezwelini kwemetronidazole. Uphenyo lokubheka ukungezweli kwimetronidazole yeTrichomonas vaginalis luveze ukuthi ukunqwabelana kwe-ayoni kwama-30 μM nama-60 μM kwaholela ekulahlekelweni kokungezweli kwamanye ama-ayisoleythi. Ngaphezu kwalokho, ukuhlaziya okuchazwa wulibofuzo kukhombisa ukuthi i-ayoni idlale indima enkulu ekuhlaziyeni okuvezwa wukungezweli okuhlobene nolibofuzo. Kodwa, izakhi ze-antimicrobial ye-ayoni ekukhuleni kweTrichomonas vaginalis zazibonakala, okwaba nomthelela ebunjalweni bokuguquka kokuveza ulibofuzo. Ukusebenza ngendlela kwamananoemulsions asuselwa ezitshalweni uma kuqhathaniswa neTrichomonas vaginalis kwacaciswa. Ukungezweli okugudlukile kwenanoemulsions ukubhekana nokutheleleka ngendlela encike ekunqwabelaneni okwehla kusuka ku-4 μg/ml kuya ku-1 μg/ml, okuthembisa ukubuyisela endaweni kokusebenza ngendlela kokwelapha okugxiliswe ezitshalweni. Okokugcina, ukugayeka kwezinhlayiyana ze-ayoni efafazwe ngechitosan ne-RNA engenelela kancane, ismall interfering RNA (siRNA) ukuthulisa ulibofuzo nengxubekwelapha ehlosiwe kwiT. vaginalis kwethulwa. Lezi zinhlayiyana zagayeka ngempumelelo zaphinde zahlangana ngendlela ne-siRNA, okunika ukungenelela kwengxubekwelapha okuwumzamo. Imithelela egxile ekunqwabelaneni kokusebenza kwamaseli yabonakala, okugqamisa

isidingo sokukhulisa insebenzongqo yemiqondosu yengxubekwelapha. Le miphumela ngokuhlangana imele imiqondosu eminye ebhekene nokungezweli kwemetronidazole bese kuba nophawu lwesinyathelo esimqoka ekuqhubekeleni phambili kwemithi yokwelapha nengxubekwelapha ehlosiwe yokutheleleka ngeTrichomonas vaginalis.

Isiphetho

Okutholakele jikelele kulolu cwaningo kuhlinzeka ubufakazi obuphoqayo bezinye izindlelasu ukubhekana nokungezweli nemetronidazole kwiTrichomonas vaginalis. Umumo ongxenyeningi wale thisisi uveza ubunkimbi bokungezweli kwemetronidazole nokukwazi ukwenza izingxubekwelapha ezimzamomusha. Lokhu okutholakele kuchaba indlela yokuqonda okuphelele kokutheleleka nge-Trichomonas vaginalis nezindlela zokungezweli. Kuthembisa imithi ekaliwe, ukulethwa kwemithi yokwelapha, ukucima ulibofuzo, nengxubekwelapha ehlosiwe. Kodwa, imiphumela yocwaningo ukuhumusha izingxubekwelapha zokwelapha eyiyo, ukuphenya, ukuhlaziya i-in vivo, nocwaningo lokwelapha olukhulu kubalulekile. Lezi zinyathelo zibalulekile ukubhekana nezinselelo ezikhulayo ezilethwa wukutheleleka yi- T. vaginalis nokungezweli kwemetronidazole ngesikhathi kuqinisekiswa ukuqhubeka kokungakhethwa kukho ngokwelapha endimeni yokwelashwa komphakathi.

1. CHAPTER ONE

1.1. Thesis overview

1.1.1 Thesis structure

This thesis is structured according to the guidelines stipulated by the College of Health Sciences, University of KwaZulu-Natal for the thesis by manuscript format. Each manuscript is formatted according to the journal guidelines to which they were submitted. The thesis is guided by the following chapters:

Chapter 1: This chapter is a review of the literature on *T. vaginalis* and it describes the problem statement, rationale, aims and objectives of the study.

Chapter 2: This chapter is titled "Investigating **the Associations between *Trichomonas vaginalis* and its endosymbionts (*T. vaginalis virus* and *Mycoplasma hominis*) in relation to metronidazole resistance**". This study investigated the endosymbiosis between *T. vaginalis* viruses (TVVs) and intracellular *M. hominis* in isolates of *T. vaginalis* and their potential role in metronidazole resistance.

Chapter 3: This chapter is entitled **"The role of iron on the growth and metronidazole activity in *Trichomonas vaginalis*"**. This study examined the impact of iron on the regulation of genes associated with metronidazole resistance in *T. vaginalis* isolates, highlighting the potential influence of iron supplementation on metronidazole susceptibility patterns.

Chapter 4: This chapter is titled "**Synergistic Strategies: Harnessing Plant Nanoemulsions to Combat Metronidazole-Resistant *Trichomonas vaginalis* in South Africa**". This study investigated the use of plant-based nanoemulsions as novel therapeutic agents against metronidazole-resistant *T. vaginalis* isolates, with a focus on three medicinal plants: *Ocimum tenuiflorum*, *Moringa oleifera*, and *Azadirachta indica.*

Chapter 5: This chapter is titled "**Nanocomplex-Based siRNA Delivery: Expanding Frontiers in *Trichomonas vaginalis* Infection Control and Precision Medicine**". This research explored the potential of biologically synthesized iron nanoparticles modified with chitosan and small interfering RNA (siRNA) as innovative therapeutics against *T. vaginalis* isolates.

Chapter 6: In this final chapter the relevant findings of the project in context with literature are discussed. The limitations are discussed and the conclusions emanating from this study are also presented.

1.1.2 Study design and methodology

This thesis employs a retrospective laboratory-based study design to investigate critical aspects of *T. vaginalis* biology, drug resistance mechanisms, and novel therapeutic approaches. The study encompasses various experimental components, including the retrieval and culturing of *T. vaginalis* isolates from a previous cross-sectional study, detection of endosymbionts of *T. vaginalis* by use of polymerase chain reaction (PCR) assays and exploration of the role of iron in drug resistance and the growth of *T. vaginalis* by classical culture experiments. Furthermore, this research performed gene expression of metronidazole resistance determinants using quantitative PCR assays in order to identify key genetic factors contributing to drug resistance. Lastly, this research explored the use of nanomaterials synthesized from plant extracts and their impact on *T. vaginalis*, as well as their cytotoxicity on vaginal epithelial cells. The comprehensive approach of this study design contributes valuable insights into the biology and therapeutic options for *T. vaginalis*, paving the way for potential advancements in the understanding and management of this prevalent sexually transmitted infection (STI).

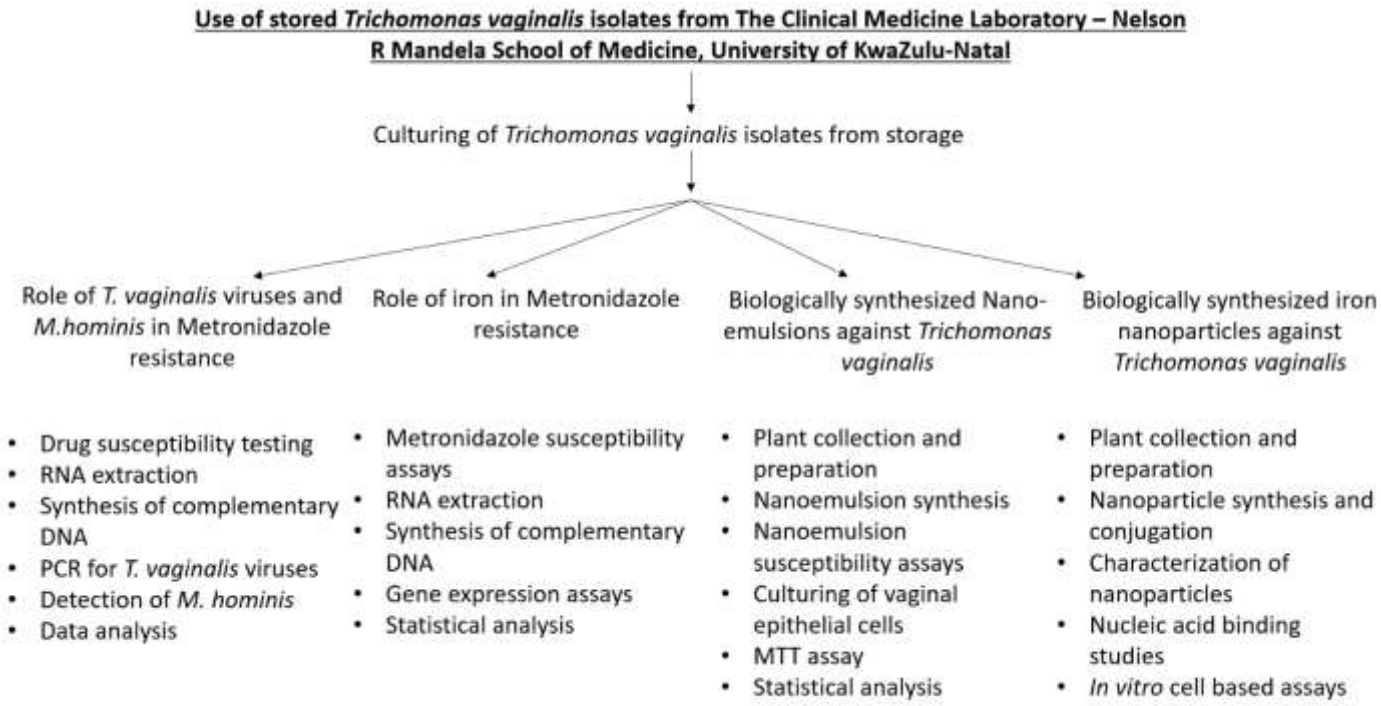

Figure 1: Overview of the thesis methodology

1.2 Introduction

Trichomonas vaginalis is considered a silent but potent threat to global public health. As a parasitic protozoan responsible for trichomoniasis, it takes centre stage in the realm of sexually transmitted infections (STIs), holding the dubious distinction of being the most common non-viral STI worldwide (Centers for Disease Control and Prevention (CDC), 2022; World Health Organization (WHO), 2021). Recent estimates provided by the WHO in 2021 reveal a staggering 156 million new trichomoniasis infections, underscoring the ubiquity of this often overlooked but pervasive infection (WHO, 2021). *T. vaginalis* is an obligate human parasite, finding its only known host in humans and predominantly transmitting via sexual intercourse (Phukan *et al.,* 2013). The longevity of *T. vaginalis* infection differs between genders, with women enduring the infection for extended periods, ranging from months to years, whereas the infection typically subsides in men within a relatively brief span of less than ten days (Fletcher *et al.,* 2012).

While trichomoniasis remains asymptomatic in a significant proportion of women, its symptomatic presentation can encompass a range of distressing genital signs and symptoms (Kissinger, 2015). These may include copious vaginal secretions accompanied by a malodorous character, inflammation of the vagina often accompanied by sensations of burning and itching, and the presence of yellow to greenish secretions (Kissinger, 2015). Symptoms may further manifest as redness and inflammation in the vulvar and surrounding areas, frequent urination accompanied by pain, dysuria, urethritis, cystitis, cervicitis, itching, and burning in the vulvar region, dyspareunia, swollen labia, and vaginal secretions Interestingly, age appears to play a role in the likelihood of *T. vaginalis* infections, with older women demonstrating an increased susceptibility (Graves *et al.,* 2020).

The diagnosis of *T. vaginalis* can be carried out through microscopic examination of a wet mount of vaginal fluid or by employing highly sensitive nucleic acid amplification tests (NAAT) (Schwebke *et al.,* 2018). The treatments recommended by the CDC and WHO for *T. vaginalis* infections consist of metronidazole and tinidazole (WHO, 2021; CDC, 2022). Due to an increase in reports on

metronidazole-resistant *T. vaginalis* strains, the efficacy of this longstanding treatment regimen is threatened (Cudmore *et al.,* 2004). Several studies have uncovered intriguing factors that contribute to metronidazole resistance in *T. vaginalis*, including the influence of *T. vaginalis* viruses (TVVs) and the potential impact of coinfection with *M. hominis* (Graves *et al.,* 2019; Margarita *et al.,* 2022). *T. vaginalis* viruses, a family of non-segmented RNA viruses, have complex interactions with *T. vaginalis* and can influence various aspects of *T. vaginalis* pathogenesis and the host immune response. These viruses have been associated with genital symptom severity, including dysuria, erythema, and vaginal discharge. Moreover, iron, a pivotal element in the biology of *T. vaginalis*, plays a significant role in regulating genes associated with metronidazole resistance (Argáez-Correa *et al.,* 2019). The competition between metronidazole and hydrogenase for electrons within the hydrogenosome, the organellar compartment responsible for metronidazole activation, is influenced by iron availability (Hrdý *et al.,* 2005). Alterations in iron-dependent processes have been linked to resistance development, further emphasizing the role of iron metabolism in metronidazole resistance.

Nanoparticles synthesized from traditional plants, offers an affordable, sustainable, and accessible alternative therapeutic for *T. vaginalis* infections. In tandem, this research investigates the use of small interfering RNA (siRNA) as a targeted gene delivery method to disrupt specific molecular pathways in *T. vaginalis*, aiming to negate resistance development and reduce the virulence of this pathogen.

1.3 Literature Review

1.3.1 Overview of the parasite *Trichomonas vaginalis*

1.3.1.1 Epidemiology

T. vaginalis is an anaerobic flagellated protozoan parasite that colonizes predominantly the squamous epithelium of the urogenital tract, causing a very common sexually transmitted disease called trichomoniasis (1) *T. vaginalis* is the most prevalent non-viral STI (2). According to the WHO, there are 276 million new cases of this infection each year, and of these, approximately 90% are among

those individuals living in resource-limited settings (3). *T. vaginalis* affects both men and women, with a global prevalence being estimated to be approximately 8.1 % and 1.0 % in women and men, respectively (4). A higher incidence of *T. vaginalis* has been seen in sexually mature females (5). This parasitic microorganism infects the vaginal and vulvar sites in women and the urethra in both genders (6). It is easily transmitted between sexual partners during penile–vaginal sex, which is the leading route of transmission (7). There are currently no surveillance programs in place for *T. vaginalis*, thus its epidemiology is not completely understood. However, it is known to vary drastically by population and geography (1).

1.3.1.2 Characteristics of T. vaginalis

T. vaginalis is known as a monogenetic parasite, with humans being their natural hosts (8). This colourless pyriform flagellate exist only as a trophozoite phase and does not contain a cystic phase (Figure 2) (9). It contains a single nucleus at the anterior. Within the cytoplasm there are numerous cytoplasmic granules and parabasal bodies (9). It is not an active parasite and exhibits non-directionally jerky movement (9). Although the exact mode of nutrition is not well known, Wenrich (1947) did observe that this parasite ingests particle substances along with bacteria, starch and erythrocytes by amoeboid movement of its cytoplasm (10). *T. vaginalis* reproduces longitudinally using binary fission with a direct life cycle and proliferation of this pathogen occurs when the acidity of the vagina shifts or becomes abnormal (9). Direct infection does not require a cystic phase or additional phases of the parasites' life cycle, but simply the larvae, which can be divided longitudinally without passing through any transitional stages (11).

Upon infection, *T. vaginalis* has an incubation period of four days to three weeks, with symptoms ranging from less dangerous to severe infection in both men and women (12). Some of the more serious infection symptoms in women include abundant vaginal secretions with a foul odour, inflammation of the vagina accompanied by burning, itching and yellow to greenish secretions, and redness and inflammation around the labia and its surrounding areas (13). Common symptoms include frequent

urination with pain, dysuria, urethritis, cystitis, cervicitis, itching and burning of the vulvar region, dyspareunia, swollen labia, and vaginal secretions (14).

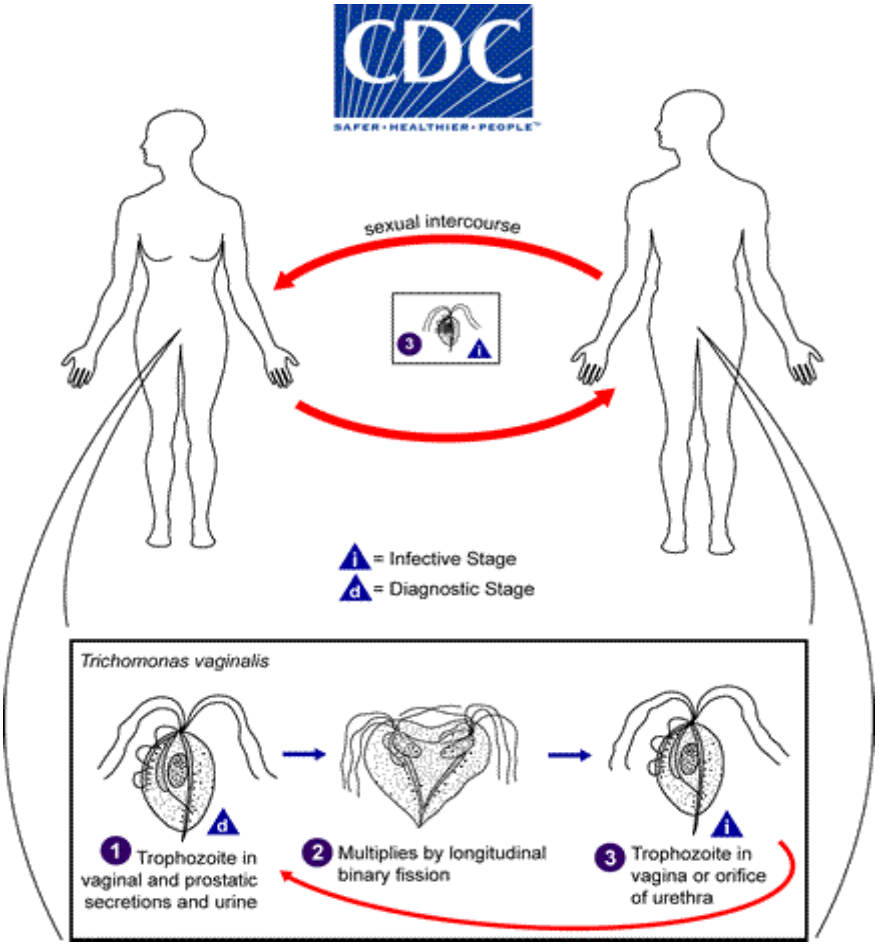

Figure 2: Life cycle of *T. vaginalis* adapted from Al-Ethafa, 2021 (9)

1.3.1.3. Management and Treatment

The current treatment of choice for *T. vaginalis,* for the past four decades, is metronidazole (15). This drug, along with its related compounds, tinidazole and seconidazole belong to the 5-nitroimidazole drug family and has previously had a 95% success rate in curing *T. vaginalis* (16). The mechanism of action of these drugs involves the generation of nitro radicals, which subsequently leads to DNA damage and cell death (17). *T. vaginalis* cells *utilize pyruvate-ferredoxin oxidoreductase (PFOR)* and *ferredoxin* linked enzymes to oxidize acetate from *pyruvate* via *acetyl coenzyme A* to gain *ATP*.

However, in the presence of nitroimidazole, the electrons from *ferredoxin* are captured by the nitro groups and nitro radicals are thus generated, which causes cell death (17). The CDC together with the WHO have developed a set of guidelines for the treatment of this infection, such as 2 g of metronidazole or tinidazole as a single dose or 400-500 mg metronidazole administered for 7 days (18). In the case of a single dose of metronidazole being missed, a single dose of tinidazole can be administered or alternatively 7 days of metronidazole can be administered (19). If this treatment protocol fails and there is no sexual re-exposure experienced, then testing should be done to determine if the strains of *T. vaginalis* have developed resistance (20).

1.3.1.4. Persistent Infections and Metronidazole resistance

T. vaginalis repeat infections have an incidence of 5%, which is high (21). Possible reasoning for retest positives includes reinfection from an untreated or infected baseline partner, an infection from a new sexual partner, or possible treatment failure (22). The probable causes of early repeat infections include drug resistance and non-adherence to treatment (23). To date, numerous metronidazole resistance strains have been documented in America, Europe, Russia and Africa (24). The resistance to metronidazole can be studied by growing *T. vaginalis* strains in the presence of different drug concentrations under either aerobic or anaerobic conditions (25). The resistant strains are dependent on either a transformed conformation of the hydrogenosome, a reduced quantity of intracellular *ferredoxin*, a reduced *pyruvate ferredoxin oxidoreductase (PFOR)* enzyme activity or a *ferredoxin* with an extraordinary redox potential (26). The fermentation of pyruvate in the cytosol instead of the hydrogenosome results in the inhibition of 5-nitoimidazole drugs (27). Additionally, the role of iron and *T. vaginalis* viruses have been identified in contributing to the pathogenesis and increase in metronidazole resistance in *T. vaginalis* (27) *T. vaginalis* has a high iron dependency, as it favours its growth and regulates some of the parasite's virulence properties. With regards to *T. vaginalis* viruses, these are acknowledged to produce phenotypic alterations that may contribute to the virulence of *T. vaginalis*, and aid in its ability to evade the host immune response.

1.4. Factors associated with metronidazole susceptibility patterns

1.4.1. Iron metabolism

Iron metabolism has been recognized for being of great importance in several microorganisms, especially for the activity of diverse antimicrobial agents and for the evolution of antimicrobial resistance (28). Iron metabolism plays a vital role in antimicrobial activity, in aerobic and microaerophilic habitats. It alters the drug-mediated redox stress, which subsequently results in oxidative damage through the Fenton reaction via the formation of highly reactive hydroxyl radicals or iron oxo intermediates (29). *T. vaginalis* has a high iron dependency, as it favours its growth and regulates some of the parasite's virulence properties (30). Numerous studies have demonstrated that iron supplementation results in an increased sensitivity of the parasite to metronidazole in both the resistant and susceptible strains (31). Iron uptake therefore plays an important role, as metronidazole co-administered with agents that may increase the intracellular iron content to increase and maximize treatment efficacy (32), could possibly serve as a potent therapeutic in clinical views.

1.4.2. Endosymbionts

The intriguing correlation between the presence of concurrent *T. vaginalis* viruses (TVV) and metronidazole resistance in *T. vaginalis* has been extensively documented in prior research (33). These unique linear double-stranded RNA viruses (dsRNA), characterized by sizes ranging between 4.5 to 5 kilobase pairs (kbp), have been unambiguously identified within *T. vaginalis* isolates, representing a remarkable revelation as the first dsRNA viruses ever recorded in the realm of protozoans (34, 35). This revelation brings into focus the remarkable interplay between TVV and *T. vaginalis*, wherein conspicuous associations have been unveiled between the presence of TVV and the intricate regulation of cysteine proteinases of *T. vaginalis*. These enzymes, instrumental in a variety of cellular processes, including cytotoxicity, cytoadherence, and the breakdown of basement membrane components

produced by vaginal epithelial cells, appear to be profoundly influenced by TVV presence, thereby opening a new avenue of inquiry into the complex web of interactions within this parasitic system (36).

Notably, the enigmatic relationship between TVV and metronidazole resistance takes on even greater significance, as *T. vaginalis* strains that bear the mark of TVV have demonstrated an unexpected shift in their response to the antiprotozoal drug metronidazole. These findings suggest that TVV might be instrumental in potentially reversing metronidazole resistance, a promising avenue for future exploration (39). Moreover, comprehensive investigations have unveiled compelling correlations between the existence of TVV within *T. vaginalis* and the manifestation of specific clinical symptoms in infected individuals. These symptoms, including dysuria, erythema, and vaginal discharge, appear to be more pronounced when TVV coexists with the protozoan, shedding light on the potential clinical implications of this viral presence (40).

Furthermore, the intricate web of interactions between TVV and *T. vaginalis* delves even deeper into the genetic realm, as Khoshan and Alderete's seminal work in 1994 revealed an intriguing correlation between a point mutation in the *T. vaginalis* genome and metronidazole resistance. This genetic mutation, marked by a transition from cytosine to thymidine at nucleotide position 66 within the ITS1 region flanking the 5.6S subunit of the ribosomal DNA gene, adds a fascinating layer to our understanding of how genetic mutations and drug resistance intersect within the realm of *T. vaginalis* (38).

Within *T. vaginalis*, another notable endosymbiont, *Mycoplasma hominis*, further complicates the landscape of metronidazole resistance. This symbiotic relationship, in addition to other factors, contributes to the intricate mechanisms of drug resistance in *T. vaginalis* (35). The presence of *M. hominis* underscores the need for a comprehensive understanding of the various interactions and pathways that collectively influence the protozoan's resistance to metronidazole, highlighting the importance of continued research into this multifaceted phenomenon.

1.5. Alternative Treatment

1.5.1 Nanomedicine

Nanomedicine is the application of nanotechnology for medical purposes and uses nanoparticles to diagnose, treat, monitor and control diseases (41). Nanoparticles range between 1 and 100 nm, and can vary in size depending on their function, physicochemical and biological characteristics of the nanomaterial, and the material from which they are derived (42). The potential of nanoparticles in cervical cancer therapy has shown an increase in the therapeutic index with a reduction in side effects (43). Nanomedicine can provide improved delivery of therapeutics whilst boosting efficiency, and therefore has the potential to overcome the limitations of conventional therapeutic approaches, thereby elucidating its potential for the treatment of various other diseases (44). Metal incorporated nanoparticles have received broad interest in industrial and medicinal applications thus far (45).

Nanomedicine is characterized into three fundamental groups, namely regenerative medicine, nanodiagnosis (diagnosis) and nanotherapy (controlled drug delivery) (41). A fourth group, theranostics (Figure 3), is newly emerging and combines both therapy and diagnostics, displaying promising effects for future cancer therapy (41).

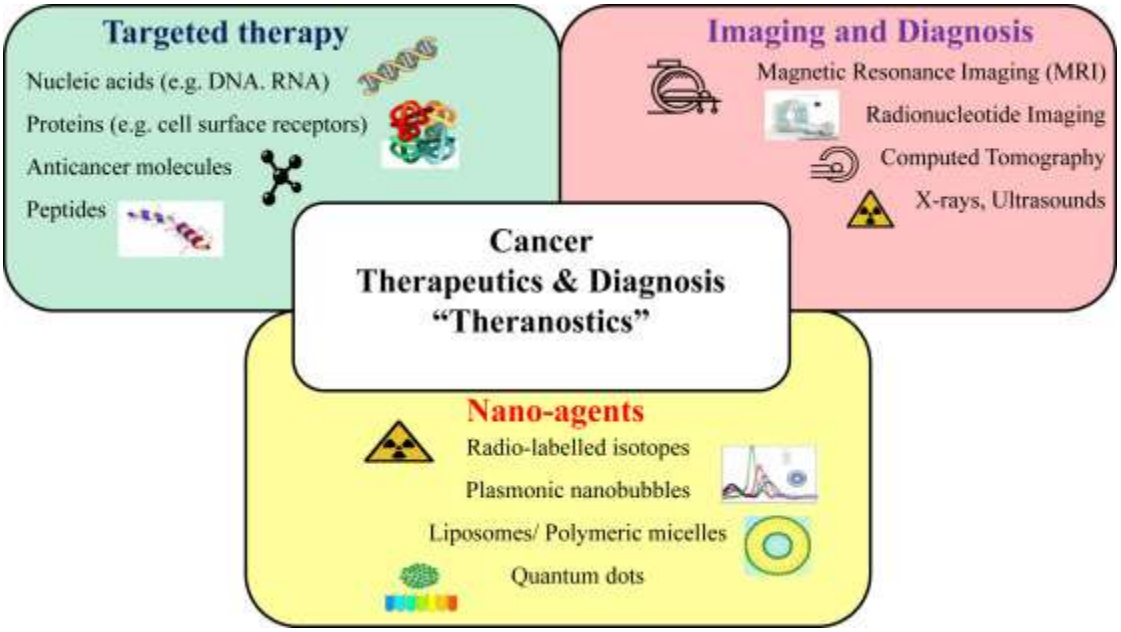

Figure 3: Application and importance of theranostics in cancer treatment Adapted from Jeyamogan, Khan and Siddiqui, 2021 (46).

Nanoparticles allow a greater distribution throughout the human body by improving movement across biological barriers, and accelerating a targeted, well-regulated, site-specific drug delivery (47). Nanoparticles have an increased surface energy and greater reactivity due to the compact, small nature of these particles, which enable their high specific surface area to volume ratio (48). Nanoparticles vary in shapes, chemical compositions and size. They also display optical, magnetic, and electric properties (42). The transition of nanomaterials across biological barriers is facilitated by a plasma biomolecule adsorption layer, called 'corona', which surrounds the surface of the nanoparticle and undergoes dynamic changes to allow the movement in areas such as the gastrointestinal tract and lungs (49). The characterization of the corona biomolecules, such as their size, shape, surface area, density, porosity, chemistry, energy, hydrophobicity, presence of ligands and valence, oxidation and conductance state, ultimately determines the interaction between the nanoparticle and the cell (50). These intrinsic properties of the nanomaterials mentioned above are exceedingly valuable in the development of pharmaceutical therapies (51).

1.5.2. Nanoparticles as potential antimicrobial agents

Extremely effective antimicrobial agents are necessitated to control the emergence of antimicrobial strains of various microorganisms such as metronidazole resistant strains of *T. vaginalis* (52). Metallic nanoparticles, especially the biologically synthesized, have shown many antimicrobial properties to date due to their unique chemical and physical properties, such as a higher reactivity and an increased surface-to-volume ratio (53). Using nanoparticles as antimicrobials is of particular importance and advantageous in that microbes have not yet developed resistance to these particles. They also do not pose acute side effects to human cells (52). These nanoparticles utilize multiple biological pathways to exert their antimicrobial effects such as inhibition of DNA, protein or enzyme synthesis, disruption of the cell wall, photo-catalytic reactive oxygen species (ROS) production and damage to cellular and viral components (54). Additionally, the synthesis of these nanoparticles is more cost-effective than the production of antibiotics. Unlike drugs, these nanoparticles can withstand much harsher conditions (e.g., high temperatures and pH), with a higher storage life. Nanoparticles have a reduced particle size, therefore the possibility of alteration in binding affinities with biological macromolecules is highly probable (55).

1.5.3 Nanoparticles in Drug Resistance

Nanoparticles combat microbial resistance through several means, such as using numerous methods to conflict the microorganisms, through nitrogen oxide liberating nanoparticles (NONPs), chitosan-enclosing nanoparticles (chitosan NPs), and metal-containing nanoparticles (56). Packaging multiple antimicrobial agents onto the same nanoparticle additionally prevents the development of resistance (57). Furthermore, existing resistance techniques have been overcome using nanoparticles, through embracing a decrease in the intake, and amplifying the release of a drug onto the surface of a microbe (58). Nanoparticles have also been used to target specific antimicrobial drugs at the infectivity spot, so

that advanced doses of the drug can be specified to the exact same spot, therefore overcoming resistance with less adverse effects on individuals (59).

1.5.4. Use of BSN for treatment of metronidazole-resistant Trichomonas vaginalis

It is important to note that there is currently limited research on the use of nanoparticles for the treatment of metronidazole-resistant *T. vaginalis* and further studies are needed to fully compare the anti-*T. vaginalis* activity of nanoparticles to that of metronidazole treatment (60). Metronidazole is a commonly used treatment for *T. vaginalis* infections, and it works by inhibiting the growth of the parasite. It has been found to be effective in the majority of cases, but resistance to metronidazole has been reported in some cases (61). Biologically synthesized nanoparticles (BSNs), on the other hand, have been found to have anti-*T. vaginalis* activity through various mechanisms, such as interference with the cell membrane and DNA damage. These nanoparticles have exhibited encouraging outcomes in combatting metronidazole-resistant *T. vaginalis* when conjugated with polymers such as chitosan, highlighting their potential as an innovative therapeutic approach (62). This conjugation strategy holds promise for addressing the challenges posed by drug resistance in *T. vaginalis* infections.

In vitro studies have shown that nanoparticles can have a similar or even better anti-*T. vaginalis* activity than metronidazole (63). However, *in vivo* studies are limited and more research is needed to fully evaluate the efficacy and safety of nanoparticles, especially BSNs as a treatment for *T. vaginalis* infections. One potential advantage of BSNs is that they are more sustainable and cost-effective than traditional synthetic drugs (64). Additionally, BSNs may have a lower toxicity profile than metronidazole, which can cause side effects such as nausea, vomiting, and allergic reactions (65). However, there are also challenges and limitations associated with the use of BSNs for the treatment of *T. vaginalis* infections, including issues related to synthesis, characterization, and delivery (66). Additionally, there is a lack of standardized methods for the synthesis and characterization of BSNs, which makes it difficult to compare results across different studies (67).

1.5.4.1. Nanotechnology in Gene delivery

Nanoparticles have recently been utilized as carriers, due to their efficient transport of diagnostic and therapeutic agents, as they are able to conquer some of the natural biological barriers (68). Barriers that are present both intracellularly and extracellularly impede gene delivery (69). These natural barriers include DNA release, endosomal escape, nuclear uptake, avoidance of the particle clearance actions, protection of the DNA (gene) from being degraded, and specific targeting of cells and/or tissues (70). These nanoparticles have the ability to gain access into numerous molecules, with pathologies or alterations in the molecules being detected in a high-throughput and a sensitive manner. Nanoparticles present greater surface area to volume ratios with reasonable magnetic, optical and biological properties. They can be engineered to deliver with improved efficacy, with respect to a specifically designed size, chemical composition, and shape (71). An ideal gene delivery vector should hold properties such as good stability, ability to evade the immune system, disruption of the endosomal membrane, ability to traverse the plasma membrane, ability to compact and defend the bound nucleic acid, and the aptitude to deliver it to the desired cell (72).

The manner in which these nanoparticles protect the conjugated nucleic acid, is dependent on the vectors' nitrogen to DNA phosphate ratios (N:P ratio), as well as the buffer solution and salt concentration (73). A number of non-viral nanosystems have been utilized (Magnetic, Metallic, quantum dots and metal oxides), each portraying varied properties (73).

1.5.4.2.Nanoparticle modification

The conjugation between metal nanoparticles and DNA can occur as a result of the essential binding of a cationic polymer (74). The main role of these polymers is to coat the nanoparticles with a positive charge so that the negatively charged DNA can be attracted to the polymer (75). Following binding, the nano-complex can pass through the cellular membrane by either a receptor-mediated or nonspecific endocytosis, as seen in Figure 4 (75).

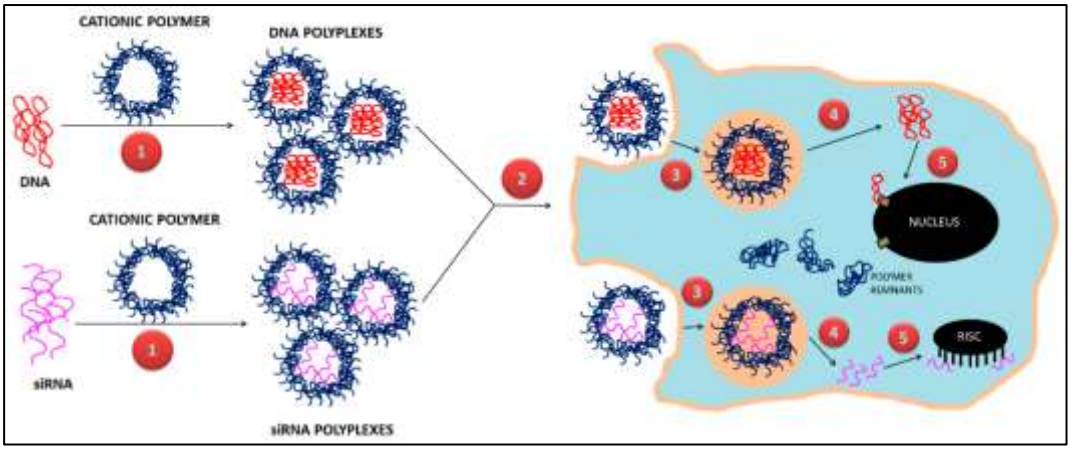

Figure 4: Nanoparticles stabilized with cationic polymers for intracellular DNA delivery to targeted cells adapted from Rai *et al*., 2019 (75). (1) DNA and siRNA complexation to the polymeric nanoparticles, (2) nanocomplexes undergoing cellular uptake, (3) entry of nanocomplex into the cell (4) release of the pharmacologically active gene from the nanoparticle, (5) transfection of DNA and siRNA, expressing the required gene by nuclear membrane transport proteins and RNA-induced silencing complex (RISC) to siRNA binding.

1.5.4.3. Combination Therapy

The potential for using nanoparticles in combination with other treatment options, such as antibiotics and antiparasitics, to enhance efficacy and reduce the emergence of resistance has been an area of active research (Figure 5) (76). Combination therapy is commonly used in the treatment of infectious diseases as it can enhance efficacy, reduce the emergence of resistance, and decrease the likelihood of side effects (77). Nanoparticles have the potential to be used in combination with antibiotics and antiparasitics to improve the effectiveness of treatment for *T. vaginalis* infections (76).

One approach to combination therapy is the use of nanoparticles to target specific pathways in the pathogen that are not affected by the other drugs. For example, nanoparticles have been shown to target the cell membrane of *T. vaginalis*, which is a different mechanism of action than that of antibiotics and

antiparasitics (76). By targeting different pathways, nanoparticles can enhance the overall efficacy of treatment and reduce the emergence of resistance.

Another approach is to use nanoparticles in combination with antibiotics and antiparasitics to improve the delivery of drugs to the site of infection. Nanoparticles can be formulated with carriers and targeting moieties to enhance their ability to reach the site of infection, thereby increasing the local concentration of drugs. This can help to overcome the issue of poor bioavailability of some drugs and increase the effectiveness of treatment (78).

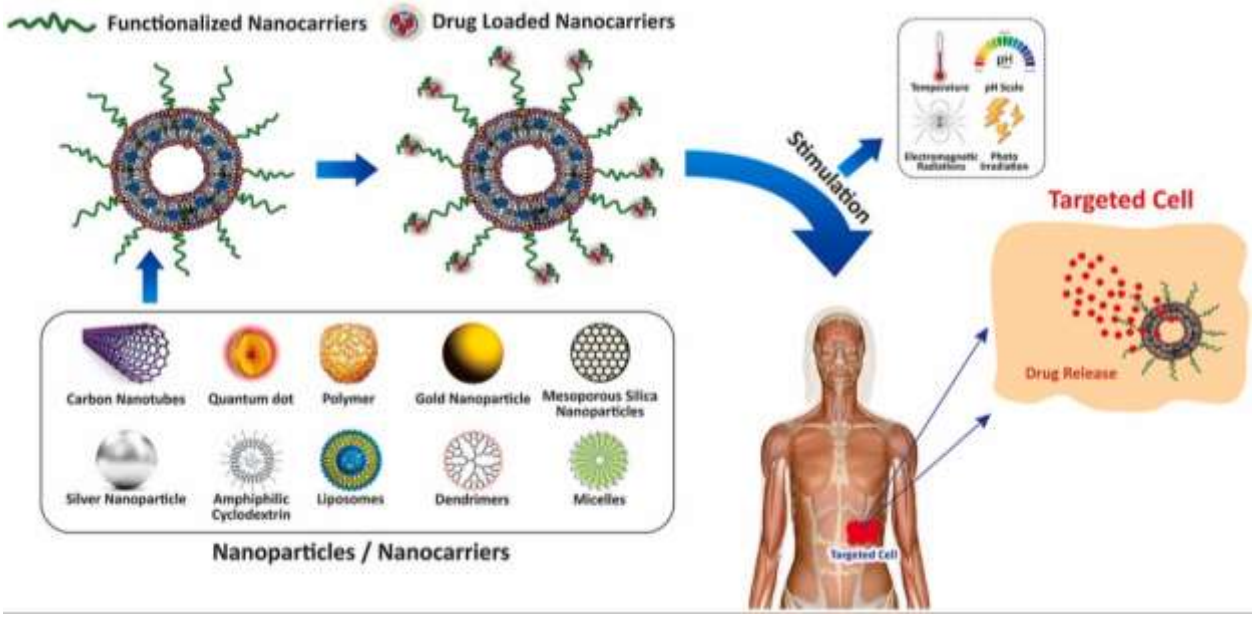

Figure 5: Targeted Drug Delivery system adopted from Shah *et al*., 2018 (79)

In addition, nanoparticles can also be used to prevent the spread of antibiotic resistance by reducing the amount of antibiotics required to achieve the same therapeutic effect (80). This can be achieved by using nanoparticles to target and eliminate the pathogen, thereby reducing the need for antibiotics (81). It is important to note that while the potential for using nanoparticles in combination with other treatment options is promising, more research is needed to fully understand the mechanisms of action and optimize the efficacy and safety of these combinations (82). BSNs are a type of nanoparticle that

are produced by living organisms, such as bacteria or yeast (83). They have been shown to have a range of potential applications, especially in medicine and biotechnology (81).

1.5.4.4. Chitosan

Chitosan is frequently utilized as a renewable carbohydrate polymer (84). Chitosan is derived through the deacetylation (removal of the acetate moiety) of chitin which is obtained from crustacean shells, such as prawns and crabs (Figure 6) (84). Naturally occurring chitin is bound to proteins and minerals, which are unwanted in synthesis reactions, and thus pre-processed through alkalization and acidification (85).

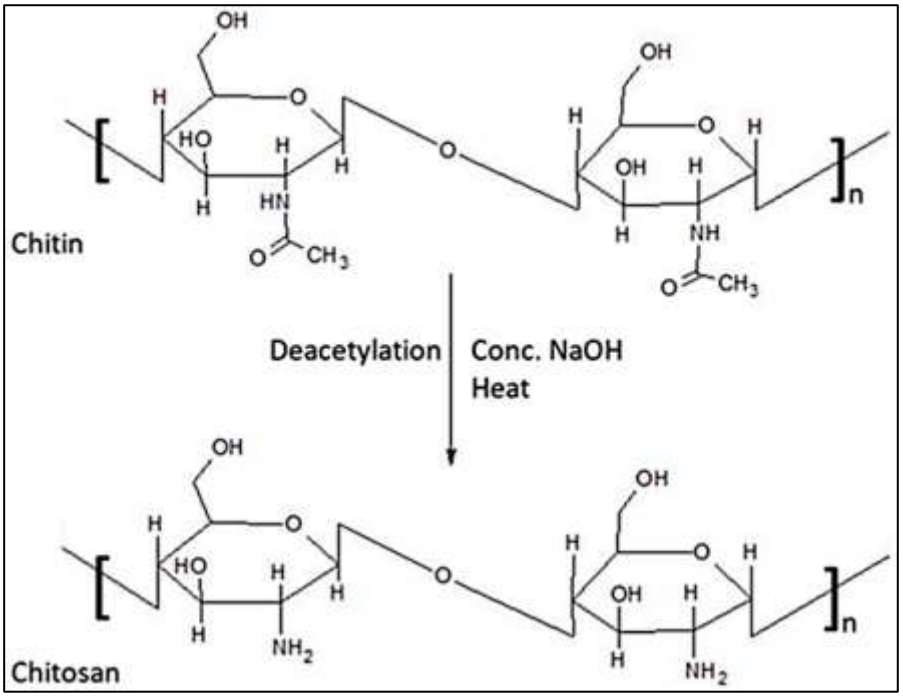

Figure 6: Chemical reaction showing the derivation of chitosan from chitin Adapted from Borchard, 2001 (84).

Chitosan is advantageous in its role in gene therapy due to its biodegradability, minimal toxicity, and its ability to relatively ease endocytosis by the host's targeted cells (85). This ease is due to the penetrative property of chitosan, since it opens the epithelial junctions and allows for the transcellular and paracellular transport of genes (84). Chitosan demonstrates the capability to be protonated under acidic conditions and thus encourages the binding of anionic nucleic acids to form a nanocomplex, by electrostatic interactions (86). The use of chitosan should be carefully matched, through specific modifications, to the preferred physical and chemical properties necessary from the polymer, as well as the biological environment that it would be subjected to prior to nanoparticle binding (86). Furthermore, chitosan can potentially enhance drug delivery to combat *T. vaginalis* by aiding in the targeted delivery of nanoparticles, increasing treatment efficacy (Figure 7). Its positive charge and mucoadhesive properties can facilitate improved nanoparticle adherence to the vaginal mucosa for more effective infection control (62).

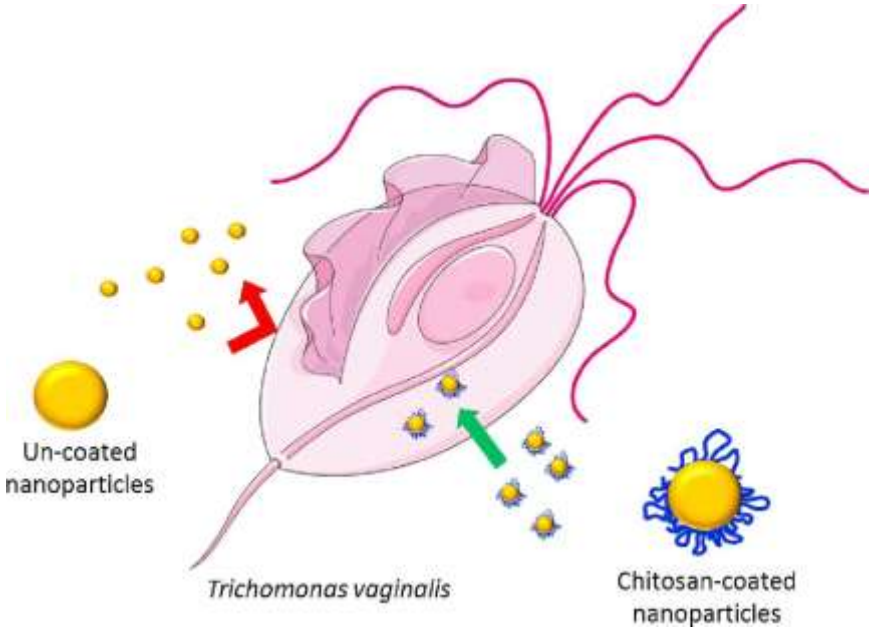

Figure 7: Treatment of *T. vaginalis* with nanoparticles Malli *et al*., 2014 (62)

1.5.4.5.Metallic Nanoparticles: Iron Nanoparticles

Numerous studies suggest the potential of iron nanoparticles to generate microbial toxicity due to their magnetic nature, which brings about a series of interactions, such as the production of reactive free radicals of Oxygen (ROS) with lipid peroxidation, DNA damage, membrane depolarization with the consequent impairment of cell integrity and the release of metal ions that affect cellular homeostasis and protein coordination (Figure 8) (87-88).

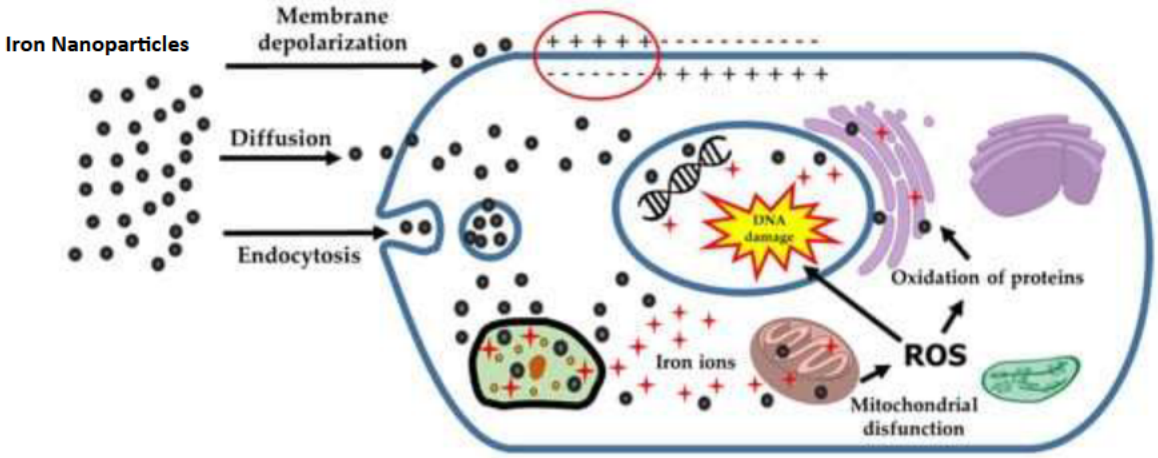

Figure 8: Mechanisms of action with which Iron nanoparticles generate cell toxicity adapted from Saleh *et al*., 2015 (88).

Iron oxide displays antimicrobial activity by generating ROS (89). These free radicals can depolymerize the polysaccharides, break DNA strands, initiate lipid peroxidation, or inactivate the enzymes (90). The antimicrobial potential of iron nanoparticles has also been identified towards microbes in the planktonic state or biofilm-forming state (91). The coadministration of amoxicillin attached to iron nanoparticles lowered the MIC for *Staphylococcus aureus* and *Escherichia coli* planktonic cells by 3 to 4 times, when compared to the drug alone. Iron nanoparticle shaves have been incorporated into medical devices, such as catheter surfaces to inhibit the colonization of

microbes (92). Additionally, these nanoparticles have displayed significant antifungal properties on different microbial species.

1.5.4.6. Synthesis of Iron Nanoparticles

Three main routes of synthesis, namely chemical, physical and biological, have been identified for iron nanoparticles. Table 1 further elaborates on these synthetic procedures.

Table 1: Various synthesis methods for Iron Nanoparticles adapted from Anjam *et al.,* 2020 (93)

No.	Method	Study Description	Procedure	Resultant iron nanoparticle size
1	Preparation of iron nanoparticles via pulsed plasma technique	The pulsed plasma in liquid is a suitable and cheap method for synthesis of nanoparticles.	The iron nanoparticles were prepared using a discharge method in which a water toluene interface is used as a medium.	≤10nm
2	Preparation of iron nanoparticles via chemical reduction method	It is a simple, reproducible and profitable method to produce iron nanoparticles.	Nanoparticles of iron were chemically synthesized by reduction of Tris (acetylacetonate) iron(III) using ascorbic acid in controlled conditions.	7±1nm
3	Iron nanoparticles via chemical vapour condensation	Iron nanoparticles produced by chemical vapour condensation (CVC) as a result of pyrolysis of organometallic Iron pentacarbonyl as a precursor. The impact of handling parameters and strengthening process on the	By using the pyrolysis of iron pentacarbonyl, the iron nanoparticles were synthesized.	6-25nm

		microstructure and size of iron nanoparticles were examined.		
4	Synthesis of iron nanoparticles via One-step reduction technique	It is an easy and simple method to synthesize iron nanoparticles. Under moderate temperature, iron nanoparticles were synthesized. Highly crystalline nanoparticles were obtained.	To obtain high crystallinity and pure iron nanoparticles at appropriate temperature (620℃), excessive citric acid is used. The citric acid and ferric nitrate were used as well.	30nm
5	Iron nanoparticles via green tea leaves extract	It is an environmentally friendly, easy scaled up and cost-effective method to synthesize iron nanoparticles. No need to use toxic chemicals, high energy, pressure and temperature.	The tea extract is prepared by adding 20g tea to 1000ml of water and heated to 80°C. The extract of tea is then collected and filtered.	116nm

The majority of the synthetic methods mentioned above requires costly instruments, high energy, physical requirements (pressure and temperature) and are potentially hazardous due to the release of toxic chemicals (94). This necessitates the need to develop a more cost-effective and eco-friendly method of synthesis from biological systems such as plants and microorganisms (95). The use of plant extracts for the biosynthesis of metal nanoparticles is advantageous when compared to other biological systems, since they eliminate the cost of cultivation and downstream processing, they produce non-hazardous waste and has a fast production process (96). The plant-mediated synthesis of iron nanoparticles improves the physicochemical and biological properties of nanoparticles (97). Iron nanoparticles tend to aggregate and sediment due to their magnetic attraction properties (98). This

aggregation can be associated with low surface charge and thus a decrease in specific surface area and reactivity of iron nanoparticles (99). However, this can be overcome by using plant-mediated synthesis where the plant extract proves colloidal stability in aqueous matrixes (100).

1.5.4.7.Biological synthesis of Nanoparticles

Biological synthesis of nanoparticles involves the use of living organisms, such as bacteria, fungi, and plants, to produce nanoparticles with specific properties and characteristics (101) (Figure 8). One of the most common methods for the biological synthesis of nanoparticles is through the use of bacteria (102). Bacteria, such as *E. coli* and *Bacillus subtilis*, have been successfully used to produce a range of nanoparticles, including silver, gold, and iron oxide nanoparticles (103, 104). These bacteria are able to reduce and stabilize metallic ions, leading to the formation of nanoparticles. The size and shape of the nanoparticles can be controlled by manipulating the conditions of the synthesis, such as the pH, temperature, and the concentration of the precursor ions (105). Another common method for the biological synthesis of nanoparticles is through the use of fungi (106). Fungi, such as *Aspergillus niger* and *Penicillium chrysogenum*, have been used to produce a range of nanoparticles, including silver and gold nanoparticles (107, 108). These fungi are able to produce extracellular enzymes, such as *laccase* and *peroxidase*, which can be used to reduce and stabilize metallic ions. Like bacteria, the size and shape of the nanoparticles can be controlled by manipulating the conditions of the synthesis (105). Plants have also been used to synthesize nanoparticles. Plant-mediated synthesis of nanoparticles involves the use of extracts from different parts of the plants, such as leaves, stem, and root, to produce nanoparticles (106). This method is considered eco-friendly, low-cost, and easy to implement (Figure 9). The plants have been found to produce a range of nanoparticles, including gold, silver, and iron oxide nanoparticles (105).

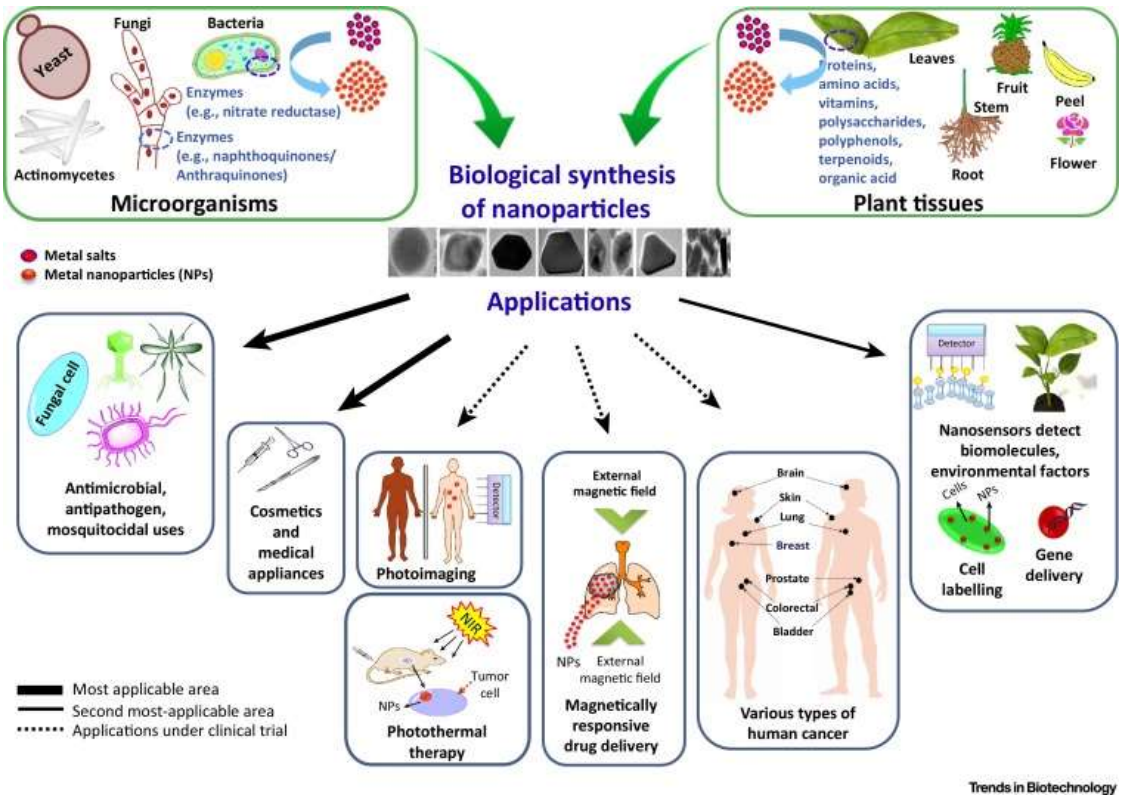

Figure 9: Synthesis of Nanoparticles adapted from Singh *et al*., 2016 (101)

1.5.5. Medicinal Plants as antimicrobial agents

Ocimum tenuiflorum, also known as 'holy basil' and 'Tulsi' in Indian languages, is commonly known for its various medicinal properties and use especially in the Ayurvedic system of medicine (109). *Ocimum* belongs to the family *Lamiaceae*, and are well known for their vast spectrum of pharmacological activities, particularly in the Indian therapeutic system, and is widely found in the Durban area (109). The leaves of *O. sanctum* hold a wide variety of compounds such eugenol, euginal and urosolic acid, carvacrol, methyl cinnamate, linalool, limatrol, caryophyllene and estragol, D-camphor, ocimin, methylchavicol, citral, linalook, nevadensin, betulinic, ursolic, oleanolic acids, beta-sitoseterol, falvanoids and nevadensin (110). These compounds from *O. sanctum* have been endorsed for treating numerous conditions including skin diseases, chronic fever, bronchitis, bronchial asthma, malaria, diarrhoea, dysentery, insect bite, arthritis and painful eye diseases (109). In addition, this plant

exhibits anticancer, antifungal, antidiabetic, antimicrobial, cardioprotective, antispasmodic, hepatoprotective, antiemetic, analgesic, adaptogenic and diaphoretic properties (110). The inhibitory role of *O. sanctum* activity against HIV-1 reverse transcriptase and platelet aggregation induced by collagen and *ADP* (*adenosine 5- diphosphate*) has recently been reported, displaying its antiviral properties (111). Due to the significant properties that this plant holds, its therapeutic role and use in this present study will be beneficial for the synthesis of iron nanoparticles, as an alternative to conventional methods.

Moringa oleifera, commonly known as *Moringa*, has gained significant attention in recent years due to its remarkable antimicrobial properties (112). This plant is rich in a variety of bioactive compounds, such as quercetin, kaempferol, and chlorogenic acid, which are known for their potent antibacterial and antiprotozoal activities (112). These compounds can disrupt the structural integrity of microbial cell membranes, inhibit their growth, and interfere with vital metabolic processes. Studies have highlighted the effectiveness of *Moringa* against a broad range of pathogens, making it a promising natural remedy for various infections, including those caused by bacteria and protozoa (113). For example, research has shown that *Moringa* extracts can effectively inhibit the growth of bacteria such as *S. aureus* and *E. coli* (114). Moreover, its antiprotozoal properties have been demonstrated against parasites such as *Giardia* and *Entamoeba* (115). These findings suggest that *Moringa oleifera* holds great potential as a natural, plant-based solution for *T. vaginalis* infections, with the advantage of being readily available and cost-effective.

Azadirachta indica, commonly known as neem, is another plant known for its medicinal properties. Neem has a long history of traditional use in various cultures, with its pharmacological activities encompassing anti-inflammatory, antifungal, and antibacterial effects (1216). Neem extracts and specific compounds, such as nimbin and azadirachtin, have demonstrated robust antimicrobial activity against a wide spectrum of pathogens. These properties have been particularly valuable in the treatment of skin infections and wounds. In the context of STIs, research has indicated that neem-derived

products may have serve as prophylactic and therapeutic agents (117). Studies have shown that neem-based formulations can inhibit the growth of various sexually transmitted pathogens, such as *Neisseria gonorrhoeae* and *Herpes simplex virus* (118). These findings suggest that *Azadirachta indica* is a promising candidate for further research into its efficacy against *T. vaginalis*. Investigating the specific mechanisms through which neem combats the parasite could unveil novel, plant-based treatments that offer alternatives to conventional therapies, with the added benefits of fewer side effects and greater accessibility.

1.5.6. Impact of BSNPs on the gut and vaginal microbiome

The impact of BSNPs on the gut microbiome is not well understood. However, some studies have suggested that they may have the potential to affect the balance of beneficial bacteria in the gut (119). For example, certain BSNPs have been shown to have antimicrobial properties, which could potentially kill off beneficial bacteria (119). Additionally, BSNPs have been shown to have the potential to induce inflammation, which could also have an impact on the gut microbiome (120).

On the other hand, BSNPs have also been shown to have the potential to act as probiotics, which are beneficial bacteria that are consumed to improve gut health (121). Some studies have suggested that certain BSNPs may be able to colonize the gut and establish themselves as beneficial bacteria Figure 10) (121).

More research is needed to fully understand the impact of BSNPs on the gut microbiome, and to determine the potential effects on beneficial bacteria. It is also important to note that the impact of BSNPs may depend on the specific type of BSNPs and the dose used.

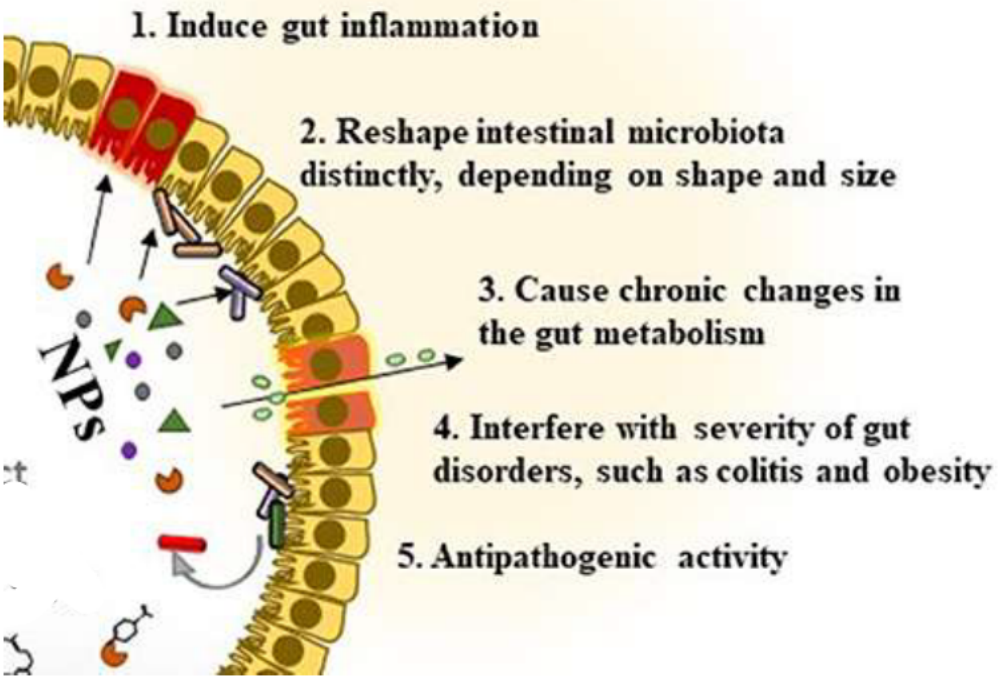

Figure 10: Modulation of Gut Microbiota by Nanoparticles adapted from Lazar *et al.,* 2018 (122)

The impact of BSNs on other vaginal microorganisms, including any potential effects on the vaginal ecosystem, is not well understood. Studies on the topic are limited (123). However, some studies have suggested that BSNPs may have the potential to affect the balance of microorganisms in the vagina, similar to their potential effects on the gut microbiome.

For example, some BSNPs have been shown to have antimicrobial properties which could potentially kill off beneficial microorganisms in the vagina. This could lead to an overgrowth of harmful bacteria, which could cause infections such as bacterial vaginosis or yeast infections (123).

Additionally, BSNPs have been shown to have the potential to induce inflammation, which could also have an impact on the vaginal ecosystem. Inflammation can disrupt the balance of microorganisms in the vagina, leading to an overgrowth of harmful bacteria and a decrease in beneficial microorganisms (124).

On the other hand, BSNPs have also been shown to have the potential to act as probiotics, which are beneficial bacteria that are consumed to improve health. Some studies have suggested that certain BSNPs may be able to colonize the vagina and establish themselves as beneficial microorganisms, which could help to maintain a healthy vaginal ecosystem (123).

1.5. Rationale

T. vaginalis is a poorly studied parasitic microorganism that has severe consequences on the health of men and women, if left untreated. The majority of trichomoniasis cases reported, are in women rather than in men, as approximately 89% of women experience symptoms. In addition, the role of iron and *T. vaginalis* viruses has been identified as contributing factors in drug resistance and the pathogenesis of this parasite. However, the mechanisms associated with drug resistance has not been studied in South African clinical isolates. *T. vaginalis* has developed increasing resistance over the years to the current treatment, metronidazole. Treatment failure has led to serious complications such as urethritis, cervicitis and vaginitis. These complications can progress to serious health consequences, such as low-birth-weight infants, preterm birth delivery, infertility, increased susceptibility to non-HIV STIs, HIV infection as well as cervical cancer. As a result of the increasing resistance to the current treatment, alternative treatment is necessary, Nanoparticles derived via green synthesis, using common medicinal plants found in South Africa, will indeed serve as an ideal candidate for the possible treatment of this

parasite. Investigating alternative therapeutics which are cheaper and eco-friendlier, will surely expand our knowledge on additional interventions that can be used in the treatment of various STIs. This study will provide insight on 1) associations between iron metabolism and the progression of metronidazole resistance 2) Correlations between *T. vaginalis* viruses and metronidazole resistance, and 3) Using plant extracts to synthesize iron nanoparticles with possible antimicrobial activity.

1.6. Aims and Objectives

Aims:

1) To determine the roles of iron and endosymbionts of *T. vaginalis* on metronidazole susceptibility patterns
2) To investigate the therapeutic potential of plant extracts against *T. vaginalis* isolates

Objectives:

(i) To investigate the influence of iron metabolism on the progression of metronidazole resistance and gene expression in *T. vaginalis*.

(ii) To explore the association between endosymbiotic *T. vaginalis* viruses (TVVs) and *M. hominis* on metronidazole susceptibility patterns.

(iii) To assess the potential antimicrobial properties of medicinal plants, specifically *Ocimum tenuiflorum* against *T. vaginalis* isolates.

(iv) To synthesize iron nanoparticles as a novel therapeutic approach for metronidazole-resistant *T. vaginalis* isolates, examining their effectiveness and biocompatibility.

(v) To investigate the utilization of small interfering RNA (siRNA) as a targeted gene delivery method for disrupting specific molecular pathways in *T. vaginalis*, aiming to reduce its virulence and mitigate resistance development.

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CHAPTER TWO

Manuscript accepted by the Journal of Infection in Developing Countries (JIDC 17592)

Investigating the Associations between *Trichomonas vaginalis* and its endosymbionts; *T. vaginalis virus* and *Mycoplasma hominis* in relation to metronidazole resistance

Rowen Govender[a,*], Nonkululeko Mabaso[a] and Nathlee Samantha Abbai[a]

[a]Department of Clinical Medicine, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa

[*]***Corresponding author:***

Nathlee Abbai

Department of Clinical Medicine,

College of Health Sciences,

Nelson R. Mandela School of Medicine,

University of KwaZulu-Natal,

Durban, South Africa

Email: 215023500@stu.ukzn.ac.za; abbain@ukzn.ac.za

ORCID Number: 0000-0003-4820-4574

Total word count: 3612

Number of Figures: 2

Number of Tables:7

Abstract

Trichomonas vaginalis (TV) is the etiological agent of the most common non-viral sexually transmitted disease (STD), trichomoniasis. *T. vaginalis* can be inherently infected with *Mycoplasma hominis* and *Trichomonas vaginalis* virus (TVV) species. Endosymbiosis of *T. vaginalis* with *M. hominis* and *T. vaginalis* viruses may contribute to metronidazole resistance in this pathogen. This study determined the prevalence of TVVs across clinical isolates of *T. vaginalis* as well as determined the symbiosis between *T. vaginalis*, TVVs and *M. hominis* in relation to metronidazole resistance.

Twenty-one clinical isolates of *T. vaginalis* were analysed in this study. The isolates were subjected to drug susceptibility assays using varying concentrations of metronidazole. Nucleic acids (RNA and DNA) were extracted from the isolates for molecular assays. The presence of intracellular *M. hominis* was determined by a *16S rRNA* polymerase chain reaction (PCR) assay with specific primers. The presence of the individual TVVs was determined by PCR using gene specific primers with template cDNA.

The prevalence of TVV and *M. hominis* were 76% (16/21) and 86% (18/21), respectively. No significant associations were observed between the presence of TVV and clinical symptoms. A significant association was noted between the coinfection of TVV4 and *M. hominis* (p=0.014). The presence of any TVV was significantly associated with metronidazole susceptibility patterns (p=0.012). No significant associations were noted between the coinfection of both endosymbionts and metronidazole resistance. The information gained here displays the ability of *T. vaginalis* to form an endosymbiotic relationship with several microorganisms, simultaneously. As per these findings, both endosymbionts pose no significant influence on metronidazole resistance.

Key Words: *Trichomonas vaginalis*; *Trichomonas vaginalis* virus, *Mycoplasma hominis*; metronidazole resistance

Key Findings:

- The occurrence of any TVV was significantly associated with metronidazole susceptibility patterns
- A significant association was observed between the coinfection of TVV4 and *M. hominis*
- No significant associations were noted between the coinfection of the endosymbionts and metronidazole resistance
- No significant associations were detected between the presence of TVV and clinical signs, and symptoms

Introduction

T. vaginalis is a parasitic protozoan and the most common non-viral STD responsible for the sexually transmitted infection (STI) trichomoniasis (CDC, 2022). In 2021, the World Health Organization (WHO) estimated that there were approximately 156 million new trichomoniasis infections (WHO, 2021). The only known host for *T. vaginalis* is humans and infection is spread by sexual intercourse (Cudmore *et al.,* 2004). In women, the infection persists for long periods of time ranging from months to even years, whereas in men they persist for less than ten days (Bradic *et al.,* 2017). Although *T. vaginalis* infection is asymptomatic in a majority of women, trichomoniasis can present with genital signs and symptoms, such as abundant vaginal secretions with a foul odour, inflammation of the vagina accompanied by burning, itching and yellow to greenish secretions, redness and inflammation around the labia and its surrounding areas (Cudmore *et al.,* 2004). Common symptoms include frequent urination with pain, dysuria, urethritis, cystitis, cervicitis, itching and burning of the vulvar region, dyspareunia, swollen labia, and vaginal secretions (Sood *et al.,* 2007). Older ages in women have also been associated with *T. vaginalis* infections (Patel *et al.,* 2018). *T. vaginalis* can be detected through either a microscopic examination of a wet mount of vaginal fluid or by highly sensitive nucleic acid amplification tests (NAAT) (Schwebke *et al.,* 2011; Hobbs and Seña, 2013; Van Der Pol *et al.,* 2014; Schwebke *et al.,* 2018). The current recommended treatment by the Centers for Disease Control and Prevention (CDC) and the WHO for *T. vaginalis* infections are metronidazole and tinidazole (Kirkcaldy *et al.,* 2012; WHO, 2022).

A double-stranded RNA (dsRNA) virus, first identified in 1985, has been observed to infect *T. vaginalis* (Wang *et al.,* 1986). This virus, termed *Trichomonas vaginalis* virus (TVV) belongs to the viral family *Totiviridae,* is non-segmented, 4.5-5.0 kilo-base pairs (kbp) and is enclosed within an 85 kilodalton major viral protein capsid in a 120-subunit icosahedral configuration (Goodman *et al.,* 2011). The TVV genetic material is replicated using viral RNA-dependant RNA polymerase. The overall protein expression of *T. vaginalis* is affected by the presence of TVV (Khoshnan *et al.,* 1994;

Provenzano *et al.,* 1997). Specific examples of this are the expression of the immunogenic protein P270 and cysteine proteinases, which are associated to host immune evasion, cytotoxicity, cytoadherence and degradation of basement membrane components produced by the vaginal epithelial cells (He *et al.,* 2017). There are four different viral strains of TVV (TVV1, TVV2, TVV3 and TVV4) which can coinfect *T. vaginalis* simultaneously, with each of these having different effects on numerous aspects of *T. vaginalis* pathogenesis (Benchimol *et al.,* 2002; Parent *et al.,* 2013). Carriers of either one of the TVVs have been suggested to upregulate pro-inflammatory host responses (Goodman *et al.,* 2011). In terms of clinical symptoms experienced with TVV, correlations between specific symptoms such as dysuria, erythema and vaginal discharge have been identified, when TVV is present in *T. vaginalis* (Flegr *et al.,* 1987). Specifically, TVV1 and TVV2 have been associated with genital symptom severity whilst TVV2 and TVV3 have been correlated with virulence factors of *T. vaginalis* and surface expression of P270 (Bessarab *et al.,* 2011; Fraga *et al.,* 2012).

Recently, a study conducted by Manny *et al*, 2022 performed viral sequence mining in publicly available transcriptomes across 60 RNA-Seq accessions that represented a minimum of 13 distinct *T. vaginalis* isolates (Manny *et al*., 2022). Thereafter, using a strategy known as *de novo* sequence assembly followed by taxonomic classification allowed for the discovery of six isolates of the newly identified fifth species known as *T. vaginalis virus 5* (TVV5) (Manny *et al*., 2022). These newly discovered isolates show high sequence identity to each other but low sequence identity to isolates of the other four species (Manny *et al*., 2022).

Additionally, some previous reports show that trichomonads harbouring TVV have shown an increased susceptibility to metronidazole (Wang *et al.,* 1987). In addition to TVV infection, some *T. vaginalis* isolates can be naturally infected with *Mycoplasma hominis*, an intracellular bacterium that usually inhabits the lower genital tract (Rappelli *et al.,* 1998). Previous studies have speculated the possible role for coinfection of *M. hominis* and TVVs in the increase of metronidazole resistance (da

Luz DB *et al.,* 1998). Additionally, strong associations between *M. hominis* coinfection and metronidazole resistance *in vitro* have been proposed (Xiao *et al.,* 2006).

It is uncertain to what extent TVV interferes with clinical response to therapy and whether it modifies trichomonal virulence. The prevalence of endosymbionts, TVV and *M. hominis*, and their association in *T. vaginalis* pathogenesis in a South African cohort is not yet known. Therefore, the aim of this study was to determine the prevalence of TVVs across clinical isolates of *T. vaginalis* as well as determine the symbiosis between *T. vaginalis*, TVVs and *M. hominis* in relation to metronidazole resistance.

Methods

Culturing of T. vaginalis isolates from storage

A total of 21 stored *T. vaginalis* isolates were available for testing in this study. These isolates were obtained from a cross sectional study which included 362 pregnant women, who were recruited during October 2018-March 2019 from the King Edward VIII hospital in Durban. The women participating in this study were 18 years and older. They were willing to provide written consent and willing to provide a self-collected vaginal swab to be tested for *T. vaginalis* infection. The laboratory testing and storage of the *T. vaginalis* isolates were performed at The School of Clinical Medicine Research Laboratory at the Nelson R. Mandela School of Medicine, University of KwaZulu-Natal. The isolates were stored at -70°C until further use. For the current study, the isolates were thawed and grown in tubes containing Diamonds TYM media (composed of 20g BBL^{TM} Trypticase Peptone, 10g Yeast Extract, 5g Maltose, 1g L-cysteine hydrochloride, 0.2g L-ascorbic acid, 0.5g Agar, 900ml distilled water and 100ml of heat inactivated donor horse serum). Diamonds TYM medium was supplemented with amikacin (4µg/ml), amphotericin (5 µg/ml), ampicillin (1 mg/ml), chloramphenicol (1 µg/ml), ciprofloxacin (2 µg/ml) and vancomycin (5 µg/ml) to prevent the growth of unwanted microbes. The inoculated media were incubated at 37°C and examined by wet mount microscopy after 24-48 hours

until axenic cultures were obtained. The axenic cultures were then subjected to drug susceptibility testing, RNA extraction and cDNA synthesis and genomic DNA extraction.

Drug Susceptibility Testing

Metronidazole susceptibility was performed in 96 well flat-bottomed microtiter plates under anaerobic incubation conditions. Two-fold serial dilutions of metronidazole was performed in Diamond's TYM medium. A standardized inoculum of $1.5x10^4$ of trichomonads/well was used in the assay. Each *T. vaginalis* isolate was added into each well, excluding the ATCC control wells. The *T. vaginalis* ATCC 50148 strain was used as a control and untreated cultures of the respective isolates were used as growth controls. Plates were incubated in air-tight anaerobic jars containing Oxoid™ AnaeroGen™ 2.5L gas pack (ThermoFisher Scientific, United States) and Oxoid™ Resazurin Anaerobic indicator strips (ThermoFisher Scientific, United States) at 37ºC for 48 hours. *T. vaginalis* motility and growth was assessed using the inverted microscope at × 400 magnification. *T. vaginalis* growth and motility was scored according to the scoring criteria described by Upcroft (Upcroft and Upcroft, 2001). Trophozoite numbers were scored 1+ (0-10 motile parasites; not more than 20% coverage of well surface and significantly less active), 2+ (20 to 50% coverage of the well surface and some trophozoite motility), 3+ (more than 50 % coverage of the well surface, almost confluent growth with much motility), and 4+ (confluent growth with full motility). The minimum inhibitory concentration (MIC) was defined as the lowest concentration of metronidazole in which a score of 1+ will be observed after 48 hours of incubation. Breakpoints suggested by Upcroft were used. MIC $\leq$ 1 µg/ml was considered susceptible, MIC = 2 µg/ml was considered intermediate (low-level resistance), and MIC $\geq$ 4 µg/ml was considered resistant. All experiments were performed in triplicate for each *T. vaginalis* isolate.

RNA Extraction

Total RNA was extracted from the cell pellets using the PureLink RNA Mini Kit (Ambion, Life technologies), according to the manufacturer's instructions. Cells were transferred to a 15 ml tube and

centrifuged at 2,000 x g for 5 minutes at 4°C to pellet the cells. The kit instructions were then followed without any modifications. The purified RNA was then stored on ice and converted immediately to cDNA.

Synthesis of complementary DNA

The extracted RNA (7µl) was converted to cDNA using the Maxima H Minus cDNA synthesis Master Mix with *dsDNase* (ThermoFisher Scientific, United States) according to the manufacturer's instructions. A nanodrop was used to measure the A_{260}/A_{280} ratio and concentration (ng/µl) of the cDNA. The converted cDNA was stored at -20°C until further use.

***PCR amplification of the T. vaginalis* viruses**

The four TVVs were amplified using the primers shown in Table 1.

Table 1: Primers used for the detection of the TVVs in the *T. vaginalis* isolates

Primer Name	Sequence 5'-3'
TVV1 forward	ATTAGCGGTGTTTGTGATGCA
TVV1 reverse	CTATCTTGCCATCCTGACTC
TVV2 forward	GCTTGAGCACTGCTCGCG
TVV2 reverse	TCTCTTTTGGCATCGCTT
TVV3 forward	AAATTAATCAACACCCTCC
TVV3 reverse	CAGATCACTTTGTGTGTC
TVV4 forward	ATGCCAGTTGCTTTCCG
TVV4 reverse	TTCCCCAATAGTTATCAG

The amplification reactions were performed using the DreamTaq 2X PCR master mix according to the manufacturer's instructions. A 25µl reaction mixture was made using the 0.5µl of the forward and

reverse primers, 10.5µl of water, 12.5µl of DreamTaq 2X PCR master mix, and 1µl of cDNA. The cycling conditions were initial denaturation at 95ºC for 3 minutes followed by 30 cycles at 95ºC for 1 minute, 55ºC for 30 seconds, 72ºC for 1 minute and a final extension at 72ºC for 5 minutes. PCR amplification was performed in a T100 thermocycler (BioRad, United States). PCR products were analysed by electrophoresis on a 1% agarose gel in 0.5X TBE buffer at 80 Volts and viewed under a UV illumination (Gene Genius System).

***DNA extraction from T. vaginalis* isolates**

The cultures were centrifuged twice for 10 minutes, thereafter cells were washed twice with phosphate buffer saline (PBS) followed by centrifugation at 1500 x g for 10 minutes. Lysis buffer (500µl) was added to the *T. vaginalis* cells and the solution was transferred into Eppendorf tubes. Tubes were then incubated at 65 ^{0}C for 30 minutes and left to cool at room temperature. DNA was then purified by adding equal volumes of phenol chloroform (1:1). The solution was centrifuged at 1500 x g for 10 minutes and the aqueous phase was transferred into a clean tube. The above-mentioned purification step was done twice. DNA was then precipitated by adding 2 × volumes of 95% ethanol and 0.1 volume of 3M sodium acetate, followed by centrifugation at 1500 x g for 10 minutes. The supernatant was discarded, and the pellet was left to dry for ± 2 hours at room temperature. Finally, the DNA was dissolved in 50µl TE buffer. The DNA purity and concentration were then measured using a nanodrop.

Detection of *M. hominis* within *T. vaginalis* isolates

Using *T. vaginalis* DNA as a template, the presence of the *16S rRNA* gene from *M. hominis* was investigated. The following *M. hominis* specific primers, forward 5'-ATACATCGATGTCGAGCGAG-3' and reverse 5'-CATCTTTTAGTGGCGCCTTAC-3' [6], were used to identify *T. vaginalis* isolates infected with *M. hominis*. PCR was performed in a total volume of 50 µl. The reaction mixture contained 16 µl of nuclease-free PCR water, 25 µl of the DreamTaq PCR Master Mix (Thermo Fisher Scientific, United States), 2 µl of each primer and 5 µl template

DNA. The cycling conditions were initial denaturation at 95ºC for 3 minutes followed by 30 cycles at 95ºC for 1 minute, 53ºC for 30 seconds, 72ºC for 1 minute and a final extension at 72ºC for 5 minutes. PCR amplification was performed in a T100 thermocycler (BioRad, United States). PCR products were analysed by electrophoresis on a 1% agarose gel in 0.5X TBE buffer at 80 Volts and viewed under a UV illumination (Gene Genius System).

Statistical analysis

Associations between *T. vaginalis* viruses and selected demographic and clinical characteristics and metronidazole resistance were examined using Chi-square and t-test statistics. All analyses were conducted using R Studio Computing.

Results

Metronidazole susceptibility patterns of the *T. vaginalis* isolates

The anaerobic MICs for metronidazole ranged from 0.25 to 4 µg/ml, and the mean MICs ± standard deviation was 1.63 ± 0.95. Of the 21 *T. vaginalis* isolates tested, 9.5% (2/21) had an MIC of 4 µg/ml (resistant), 38.1% (8/21) had an MIC of 2 µg/ml (intermediate) and 52.4% (11/21) had an MIC ≤ 1 µg/ml (susceptible) (Table 2).

Table 2: *T. vaginalis* metronidazole susceptibility results.

***T. vaginalis* isolates**	**Metronidazole MIC (µg/ml)**	**Susceptibility profile**
TV101	2	Intermediate
TV128	1	Susceptible
TV171	1	Susceptible
TV179	1	Susceptible
TV182	2	Intermediate
TV184	1	Susceptible
TV209	2	Intermediate
TV211	2	Intermediate

TV230	2	Intermediate
TV231	1	Susceptible
TV233	2	Intermediate
TV241	2	Intermediate
TV253	4	Resistant
TV266	1	Susceptible
TV270	4	Resistant
TV275	1	Susceptible
TV302	1	Susceptible
TV329	1	Susceptible
TV341	2	Intermediate
TV357	1	Susceptible
TV358	0.25	Susceptible

Prevalence of TVVs and intracellular *M. hominis*

Figure 1 shows the gel images of the individual TVVs amplified across the isolates of *T. vaginalis*. The size of the PCR amplicons was 400bp. The most prevalent TVV was TVV1 which was detected in 12 out of the 21 isolates tested (57.1%), the second most prevalent virus was TVV2, which was detected in 11 of the isolates (52.3%). TVV3 and TVV4 were detected in 3 (14.2%) and 2 of the isolates (9.52%), respectively. Additionally, 27 viruses were identified in 16 *T. vaginalis* isolates, with more than one TVV detected in ten of the isolates.

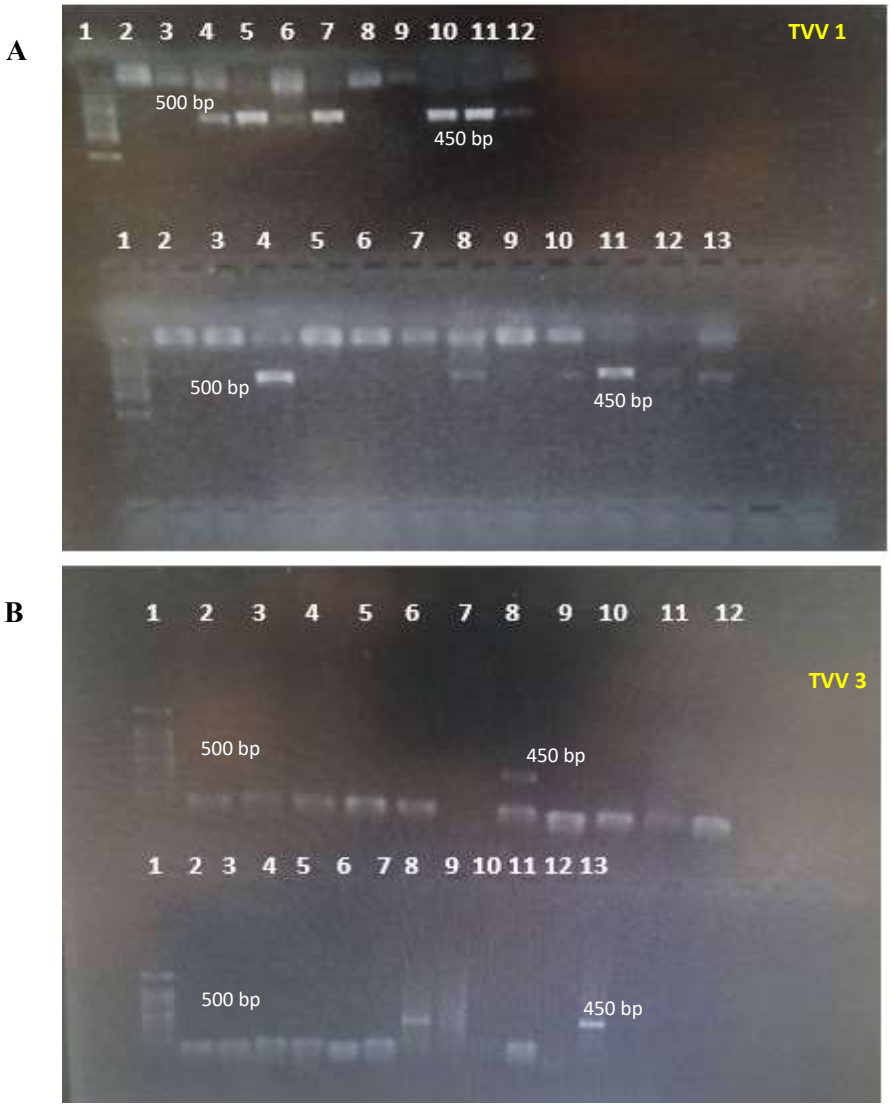

Figure 1. Gel images representing *T. vaginalis* isolates screened for TVV.

(A-first row): M: molecular marker (100 bp ladder from ThermoFisher Scientific, USA), lane 2: negative control, lanes 3-6 and 9-11: amplification of TVV1, lanes 2,7-8: no amplification of TVV1 in *T. vaginalis* isolates.

(A-second row): M: molecular marker (100 bp ladder from ThermoFisher Scientific, USA), lane 2: negative control, lanes 3,7,10-12: amplification of TVV1, lanes 2,4-6, 8-9: no amplification of TVV1 in *T. vaginalis* isolates.

(B- first row): M: molecular marker (100 bp ladder from ThermoFisher Scientific, USA), lane 2: negative control, lane 7: amplification of TVV 3, lanes 2-6, 8-11: no amplification of TVV3 in *T. vaginalis* isolates.

(B- second row): M: molecular marker (100 bp ladder from ThermoFisher Scientific, USA), lane 2: negative control, lanes 7,12: amplification of TVV3, lanes 2-6, 8-11: no amplification of TVV3 in *T. vaginalis* isolates.

Of the 21 isolates, 18 isolates harbored intracellular *M. hominis*. The expected amplicon size of 200bp corresponding to the *16S rRNA* from *M. hominis* was detected in the 18 isolates (Figure 2). The prevalence of *M. hominis* was 85.7%.

Figure 2. Gel image representing *T. vaginalis* isolates screened for *M. hominis*. M - molecular marker (100 bp ladder from ThermoFisher Scientific, USA), lane 1 - negative control (no template DNA), lanes 2-13 and 17-29 - amplification of intracellular *M. hominis* being present within the *T. vaginalis* isolates, lanes 15-16 - no amplification of *M. hominis* of intracellular *M. hominis* within the *T. vaginalis* isolates.

Association between *M. hominis* infection, presence of TVVs and metronidazole susceptibility patterns

The highest proportion of *T. vaginalis* isolates that tested positive for intracellular *M. hominis* (100%), were classified as having intermediate resistance to metronidazole when compared to 50% which were classified has being resistant and 81.8% which were classified as being susceptible. There was no significant association between the presence of intracellular *M. hominis* and metronidazole susceptibility patterns, p=0.181. This indicates that symbiosis between *M. hominis* and *T. vaginalis* does not have a contributory role towards metronidazole resistance in *T. vaginalis* (Table 3).

Overall, the presence of any one of the four TVVs was significantly associated with metronidazole susceptibility patterns, p=0.012. Despite the highest frequency of TVV1 being present in metronidazole resistant isolates (72.7%) when compared to intermediate (37.5%) and susceptible isolates (50%), this association was not significant, p=0.381. Similarly, for TVV2, TVV3 and TVV4, the highest frequencies of these viruses were shown to be present in metronidazole resistant isolates, however this was not significant, p>0.05. This indicates that symbiosis between TVVs and *T. vaginalis* does not have a significant contribution towards metronidazole resistance in *T. vaginalis* (Table 3).

Table 3: Detection of *M. hominis* and *T. vaginalis viruses (*TVV) stratified by metronidazole susceptibility patterns.

Metronidazole susceptibility patterns	Intermediate (N=8)	Resistant (N=2)	Susceptible (N=11)	p-value	Overall (N=21)
***M. hominis* status**				0.181	
Negative	0 (0.0%)	1 (50.0%)	2 (18.2%)	Fisher's	3 (14.3%)
Positive	8 (100.0%)	1 (50.0%)	9 (81.8%)		18 (85.7%)
TVV				0.012	
Negative	4 (50.0%)	1 (50.0%)	0 (0.0%)	Fisher's	5 (23.8%)
Positive	4 (50.0%)	1 (50.0%)	11 (100.0%)		16 (76.2%)
TVV1				0.381	

Negative	5 (62.5%)	1 (50.0%)	3 (27.3%)	Fisher's	9 (42.9%)
Positive	3 (37.5%)	1 (50.0%)	8 (72.7%)		12 (57.1%)
TVV2				0.120	
Negative	6 (75.0%)	1 (50.0%)	3 (27.3%)	Fisher's	10 (47.6%)
Positive	2 (25.0%)	1 (50.0%)	8 (72.7%)		11 (52.4%)
TVV3				1.000	
Negative	7 (87.5%)	2 (100.0%)	9 (81.8%)	Fisher's	18 (85.7%)
Positive	1 (12.5%)	0 (0.0%)	2 (18.2%)		3 (14.3%)
TVV4				0.581	
Negative	8 (100.0%)	2 (100.0%)	9 (81.8%)	Fisher's	19 (90.5%)
Positive	0 (0.0%)	0 (0.0%)	2 (18.2%)		2 (9.5%)

| % and p-values based on non-missing cases | * parametric p-value

Association between *M. hominis* infection and the presence of *T. vaginalis viruses* (TVV)

Overall, there was no significant association between coinfection between *M. hominis* and TVVs, p=0.549. However, when stratified by individual TTVs, a significant association was observed between TVV4 and *M. hominis* (p=0.014). Of the isolates which tested positive for TVV4, none of them had been coinfected with *M. hominis*. This shows an inverse relationship between TVV and *M. hominis* (Table 4).

Table 4: Detection of *T. vaginalis viruses* (TVV) stratified by *M. hominis* infection status.

M. hominis	**Negative (N=3)**	**Positive (N=18)**	**p-value**	**Overall (N=21)**
TVV			Fisher's, p = 0.549	
Negative	0 (0.0%)	5 (27.8%)		5 (23.8%)
Positive	3 (100.0%)	13 (72.2%)		16 (76.2%)
TVV1			Fisher's, p = 0.229	
Negative	0 (0.0%)	9 (50.0%)		9 (42.9%)

Positive	3 (100.0%)	9 (50.0%)		12 (57.1%)
TVV2			Fisher's, p = 1.000	
Negative	1 (33.3%)	9 (50.0%)		10 (47.6%)
Positive	2 (66.7%)	9 (50.0%)		11 (52.4%)
TVV3			Fisher's, p = 0.386	
Negative	2 (66.7%)	16 (88.9%)		18 (85.7%)
Positive	1 (33.3%)	2 (11.1%)		3 (14.3%)
TVV4			Fisher's, p = 0.014	
Negative	1 (33.3%)	18 (100.0%)	0.029	19 (90.5%)
Positive	2 (66.7%)	0 (0.0%)	0.029	2 (9.5%)

| % and p-values based on non-missing cases | * parametric p-value

In order to determine if previous treatment for STIs would have provided a selective pressure for the acquisition of TVVs, the association between past treatment and the presence of TVVs was determined. According to Table 5 and 6, neither the overall presence of any one of the four TVVs nor any of the individual TVVs were significantly associated with past symptoms or the past treatment of STIs ($p>0.05$). This indicates that previous exposure to infection or past treatment with metronidazole does not provide a selective pressure for the acquisition of TVVs. In addition, having current symptoms of STIs such as abnormal vaginal discharge is not significantly associated with the presence of TVVs, $p>0.05$ (Table 7). This re-iterates that clinical factors are not the drivers for the acquisition of these viruses in *T. vaginalis* isolates.

Table 5: Association between past treatment for sexually transmitted infections (STIs) and the presence of *T. vaginalis viruses* (TVVs).

Previously treated for STI in the past	**No (N=14)**	**Yes (N=7)**	**p-value**	**Overall (N=21)**
TVV			Fisher's, p = 0.624	
Negative	4 (28.6%)	1 (14.3%)		5 (23.8%)
Positive	10 (71.4%)	6 (85.7%)		16 (76.2%)
TVV1			Fisher's, p = 0.642	
Negative	7 (50.0%)	2 (28.6%)		9 (42.9%)
Positive	7 (50.0%)	5 (71.4%)		12 (57.1%)
TVV2			Fisher's, p = 1.000	
Negative	7 (50.0%)	3 (42.9%)		10 (47.6%)
Positive	7 (50.0%)	4 (57.1%)		11 (52.4%)
TVV3			Fisher's, p = 0.247	
Negative	13 (92.9%)	5 (71.4%)		18 (85.7%)
Positive	1 (7.1%)	2 (28.6%)		3 (14.3%)
TVV4			Fisher's, p = 1.000	
Negative	13 (92.9%)	6 (85.7%)		19 (90.5%)
Positive	1 (7.1%)	1 (14.3%)		2 (9.5%)

|% and p-values based on non-missing cases | * parametric p-value

Table 6: Association between past symptoms of sexually transmitted infections (STIs) and the presence of *T. vaginalis viruses* (TVVs).

Past STI symptoms	No (N=10)	Yes (N=11)	p-value	Overall (N=21)
TVV			Fisher's, p = 0.311	
Negative	1 (10.0%)	4 (36.4%)		5 (23.8%)
Positive	9 (90.0%)	7 (63.6%)		16 (76.2%)
TVV1			Fisher's, p = 0.387	
Negative	3 (30.0%)	6 (54.5%)		9 (42.9%)
Positive	7 (70.0%)	5 (45.5%)		12 (57.1%)
TVV2			Fisher's, p = 0.670	
Negative	4 (40.0%)	6 (54.5%)		10 (47.6%)
Positive	6 (60.0%)	5 (45.5%)		11 (52.4%)
TVV3			Fisher's, p = 0.586	
Negative	8 (80.0%)	10 (90.9%)		18 (85.7%)
Positive	2 (20.0%)	1 (9.1%)		3 (14.3%)
TVV4			Fisher's, p = 0.214	
Negative	8 (80.0%)	11 (100.0%)		19 (90.5%)
Positive	2 (20.0%)	0 (0.0%)		2 (9.5%)

| % and p-values based on non-missing cases | * parametric p-value

Table 7: Association between current symptoms of sexually transmitted infections (STIs) such as abnormal vaginal discharge and the presence of *T. vaginalis viruses* (TVVs).

Current abnormal vaginal discharge	No (N=13)	Yes (N=8)	p-value	Overall (N=21)
TVV			Fisher's, p = 0.325	
Negative	2 (15.4%)	3 (37.5%)		5 (23.8%)
Positive	11 (84.6%)	5 (62.5%)		16 (76.2%)
TVV1			Fisher's, p = 0.673	
Negative	5 (38.5%)	4 (50.0%)		9 (42.9%)
Positive	8 (61.5%)	4 (50.0%)		12 (57.1%)
TVV2			Fisher's, p = 1.000	
Negative	6 (46.2%)	4 (50.0%)		10 (47.6%)
Positive	7 (53.8%)	4 (50.0%)		11 (52.4%)
TVV3			Fisher's, p = 0.257	
Negative	10 (76.9%)	8 (100.0%)		18 (85.7%)
Positive	3 (23.1%)	0 (0.0%)		3 (14.3%)
TVV4			Fisher's, p = 0.505	
Negative	11 (84.6%)	8 (100.0%)		19 (90.5%)
Positive	2 (15.4%)	0 (0.0%)		2 (9.5%)

The association between current abnormal vaginal discharge and the presence of TVV was investigated and the results yielded showed that testing positive for any TVV was not associated with current abnormal vaginal discharge. However, this association was not statistically significant ($p>0.05$).

Discussion

To the best of our knowledge, this is the first study to determine the prevalence of TVVs across clinical isolates of *T. vaginalis* as well as determine the symbiosis between *T. vaginalis* isolates, TVVs and *M. hominis* in relation to metronidazole resistance in our current setting.

In this study, 76% of the *T. vaginalis* isolates tested positive for at least one of the TVVs. This high prevalence of TVVs found in this study is comparable to a prevalence of 81.9% (95% CI: 71.1–90.0) reported in Cape Town, South Africa (Weber *et al.,* 2003) and Baltimore City, Maryland, 75.0% (95% CI: 55.1–89.3) (Wendel *et al.,* 2002). More recent studies have reported lower carriage of TVV, 16% (95% CI: 7-34), in Turkey (Ertabaklar *et al.,* 2021) and a higher prevalence, 44% (95% CI: 30-58), in Italy (Margarita *et al.,* 2019). In this study, overall, 27 viruses were identified in 16 *T. vaginalis* isolates, with more than one TVV detected in ten of the isolates. *T. vaginalis* can be infected by more than one species of TVV, simultaneously (Margarita *et al.,* 2019). The aptitude of diverse viral species to cohabit a single host is common among *Totivirus* as they are predominantly non-cytopathic and can well-adjust in the host cell environment (Goodman *et al.,* 2011). Rivera *et al.,* 2017 suggested that the occurrence of numerous TVVs may be as a result of more than one *T. vaginalis* isolate infecting the same patient, as opposed to concurrent infections of different TVVs in a single *T. vaginalis* cell (Rivera *et al.,* 2017). The occurrence of numerous TVVs in a single *T. vaginalis* cell may contribute to the upregulation of inflammatory reactions and development of trichomoniasis (Margarita *et al.,* 2019). Similar to previous reports, TVV1 and TVV2 were the more dominant viruses in this study when compared to TVV3 and TVV4 (Jehee *et al.,* 2017). Bahadory *et al.,* 2021 reported in their systemic review and meta-analysis on the global status of TVV, that African regions had the highest prevalence, 66% (95% CI, 25–92%), of TVVs (Bahadory *et al.,* 2021). Accumulated evidence suggests that the endosymbiotic TVV may play a role in drug susceptibility of *T. vaginalis*, and in the modulation and influence of the protozoan's virulence (Malla *et al.,* 2011). TVV is known to alter the expression profile of host cysteine proteases, which are responsible for promoting *T. vaginalis* cytoadhesion to the vaginal epithelium and mediating cytotoxicity to aid in parasite survival, and host immune evasion (Khanaliha *et al.,* 2017). Previous studies show that *T. vaginalis* infected with TVV have a higher growth rate and are increasingly difficult to maintain in a culture (Khanaliha *et al.,* 2017). Among the *T. vaginalis* isolates in this study, it was noted that protozoa uninfected by viruses had a slower growth

rate as compared to those that contained the virus. These results are in line with those by Rivera and colleagues (Rivera *et al.,* 2017). In addition, Margarita *et al.,* 2019 noticed that their *T. vaginalis* isolates infected by all four TVVs were difficult to cultivate *in vitro* (Margarita *et al.,* 2019).

The array of clinical symptoms caused by *T. vaginalis* can differ from asymptomatic to serious symptoms. TVV have been frequently identified in symptomatic patients, however, numerous studies have found that there are no significant associations between TVV harbouring strains and clinical symptoms (Masha *et al.,* 2017; CDCP, 2022). In this study, in order to determine if previous treatment for STI would have provided a selective pressure for the acquisition of TVVs, the association between past treatment of STIs and the presence of TVVs was determined. According to the analysis, neither the overall presence of any one of the four TVVs nor any of the individual TVVs were significantly associated with past symptoms or the past treatment of STIs. This indicates that previous exposure to infection or past treatment with metronidazole does not provide a selective pressure for the acquisition of TVVs. In addition, this study showed that having current symptoms of STIs such as abnormal vaginal discharge is not significantly associated with the presence of TVVs. This is consistent with the data from previous reports that showed that clinical factors are not the drivers for the acquisition of these viruses in *T. vaginalis*. However, an association between TVV and clinical symptoms (vaginal discharge and erythematous), have been recognized by some studies (Graves *et al.,* 2019). These results may differ to the findings from the current study, due to the heterogeneity in sample number and geographical areas. Previous studies showing associations between TVV and clinical symptoms have identified that TVV1 is linked to milder symptoms of dysuria and vaginal discharge, whilst TVV2 are specifically associated with harsher symptoms when compared to those described above (Graves *et al.,* 2019). Associations between the presence of TVV and metronidazole resistance in *T. vaginalis* have been elucidated in previous reports (Graves *et al.,* 2019). This study noted that the presence of any one of the four TVVs was significantly associated with metronidazole susceptibility. However, the findings in this study evidently reveal the lack of relationship between the presence of TVV and

metronidazole resistance, as no significant association was noted. This indicates that the symbiosis between TVVs and *T. vaginalis* does not have a significant contribution towards metronidazole resistance in *T. vaginalis.*

In addition to TVV, this study evaluated the prevalence of intracellular *M. hominis* infection in *T. vaginalis* isolates and assessed whether associations exist between metronidazole resistance and co-infection with *M. hominis* and TVV. *M. hominis* can survive, multiply and invade the cytoplasm of *T. vaginalis*, aiding in its defence mechanism during human infection (Dessi *et al.,* 2006). In this study, 86% of the *T. vaginalis* isolates tested positive for *M. hominis* and of this, 72% were coinfected with TVV. Previous reports have described varying prevalence rates of intracellular *M. hominis* in symbiosis with *T. vaginalis*, based on different geographical regions. These range from as high as 92.5% in Mozambique, Angola and Italy to as low as 20% in the United States of America (Rappelli *et al.,* 2001). The symbiosis of *M. hominis* in association with *T. vaginalis* has significant clinical consequences and correlations with metronidazole resistance of *T. vaginalis*. Majority of the *T. vaginalis* isolates infected with *M. hominis* were susceptible to metronidazole. This finding is similar to previous literature that showed no association between metronidazole resistance and *M. hominis* infection (da Luz *et al.,* 2015). In terms of the coinfection of both *M. hominis* and TVV, this study demonstrated that 72.2% of the *T. vaginalis* isolates harbouring *M. hominis* were infected by at least one subclass of TVV, however this association was not significant. Contrary to this, statistical correlation and significance was demonstrated between the presence of *M. hominis* and TVV4. Of the isolates that tested positive for TVV4, none of them had been coinfected with *M. hominis*. This shows an inverse relationship between TVV and *M. hominis.* This study further showed that none of the *T. vaginalis* isolates were devoid of either *M. hominis* or TVV. This validates the aptitude of *T. vaginalis* to adapt to symbiosis with different microorganisms to aid in its' pathogenesis.

The influence of the aforementioned endosymbionts on *T. vaginalis* resistance to metronidazole was assessed. Previous studies have elucidated the positive correlations between infection by either TVV

or *M. hominis* with metronidazole resistance (Snipes *et al.,* 2000). However, some reports showed a lack of correlation between the endosymbionts and metronidazole resistance (Dessi *et al.,* 2006). Similarly, this study showed no significant association between the presence of intracellular *M. hominis* and metronidazole susceptibility patterns. Furthermore, 81.8% of the *T. vaginalis* isolates that tested positive for *M. hominis*, were susceptible to metronidazole. No significant associations were seen in *T. vaginalis* isolates infected with both endosymbionts in terms of metronidazole resistance. This indicated that the endosymbiosis between *M. hominis* and TVV does not have a contributory role towards metronidazole resistance in *T. vaginalis* isolates. The finding in this study advocates the importance of further large-scale studies to provide associations between metronidazole resistance in *T. vaginalis* isolates and the presence of both TVV and *M. hominis*.

Conclusion

In conclusion, the prevalence of TVV in this study showed that majority of the *T. vaginalis* isolates were infected with TVV. The study was limited by the small number of isolates screened for TVV, which made it challenging to assess any associations with TVV carriage on clinical signs and symptoms. TVV was not associated with clinical symptoms or metronidazole resistance. These results advocate that TVV and *T. vaginalis* may have a commensal relationship. Regardless of the low number of *T. vaginalis* isolates, the presence of all individual and mixed (coinfections) TVV subtypes were detected in our isolates. This demonstrated that there is prominent genetic diversity of TVV in the *T. vaginalis* isolates from women in Durban, South Africa. Additional studies with a higher number of isolates should be conducted to substantiate these results. The data obtained showed the high prevalence of *T. vaginalis* isolates infected with different types of TVVs as well as *M. hominis*, displaying the aptitude of *T. vaginalis* to establish an endosymbiotic relationship with numerous microorganisms, simultaneously. The presence of *M. hominis* in *T. vaginalis* isolates had no association with TVV or metronidazole resistance. The findings in this study suggests the necessity for more cohort and cellular studies to intensely comprehend the interaction between *T. vaginalis*,

TVV and *M. hominis,* and assess how they influence pathogenicity, occurrence of symptoms and modification of host responses. This suggests a new field of research to explore.

Author Contributions

Conceptualization, NA and RG; writing—original draft preparation, RG; writing—review and editing, NA and RG; supervision, NA. All authors have read and agreed to the published version of the manuscript."

Conflicts of Interest

The authors declare no conflict of interest.

Funding: National Research Foundation, College of Health Sciences funding (University of KwaZulu-Natal)

Ethical Standards

Ethical approval was obtained from the Biomedical Research Ethic Committee (BREC) (BREC/00003557/2021).

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CHAPTER THREE

The manuscript is under review in the journal *Parasitology* (PAR-2023-0096).

The role of iron on the growth and metronidazole activity in *Trichomonas vaginalis*

Rowen Govender[a,*] and Nathlee S Abbai[a]

[a]Department of Clinical Medicine, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa

[*]***Corresponding author:***

Rowen Govender

Department of Clinical Medicine,

College of Health Sciences,

Nelson R. Mandela School of Medicine,

University of KwaZulu-Natal,

Durban, South Africa

Email: 215023500@stu.ukzn.ac.za ; ABBAIN@ukzn.ac.za

ORCID Number: 0000-0003-4820-4574

Abstract

Background

The drug metronidazole and its related 5-nitroimidazole derivatives, have proven effective against various pathogens such as *Trichomonas vaginalis* (TV). Studies have also shown that *T. vaginalis* can hinder and restrict metronidazole activation due to numerous down-regulated genes occurring in resistant isolates and alterations in iron-dependent processes. This study assessed the role of iron on the regulation of genes associated with metronidazole resistance in *T. vaginalis* isolates from South Africa. This study also investigated the association between iron supplementation and metronidazole susceptibility patterns in the *T. vaginalis* isolates.

Methods

Eleven stored *T. vaginalis* isolates from self-collected vaginal swabs obtained from antenatal women attending care at the King Edward VIII in Durban, South Africa were used. Metronidazole susceptibility assays were performed in the presence of iron using media containing different increased concentrations of $FeSO_4$ (0 μM, 30 μM, 60 μM, and 120 μM). Susceptibility of trichomonads to metronidazole was determined *in vitro*. The viability of the cells was determined by the trypan blue exclusion assay. The fold change in expression of target genes (*PFOR, flavin reductase 1, ferredoxin* and *nitroreductases 4* and *6*) was assessed using the PowerUP SYBR Green Master Mix (ThermoFisher Scientific, United States). The formula used to calculate the expression fold exchange was $\Delta\Delta C_T = \Delta C_{T\text{treated sample}} - \Delta C_{T\text{reference sample}}$.

Results

In this study, an iron concentration of 120uM resulted in the death of all viable trophozoites. In the absence of iron, 9.09% (1/11) of the isolates were susceptible to metronidazole, 72.7% (8/11) had an intermediate susceptibility profile and 18.2% (2/11) were resistant to metronidazole. However, increasing concentrations of iron (30uM and 60uM) resulted in the loss of the resistance phenotype.

With respect to the gene expression analysis, the individual genes were not expressed in the susceptible isolate. For the resistance isolates, the expression of *PFOR, flavin reductase 1, ferredoxin* and *nitroreductases 4* and *6* was higher in the presence of iron when compared to expression in the absence of iron. However, there was no statistical significance in the expression of the genes in the presence and absence of iron, $p>0.05$.

Conclusion

The presence of iron resulted in an increase in the expression of resistance genes, but as expected this was not significant due to the antimicrobial properties of iron on the growth of *T. vaginalis* as shown in this study. Future studies are needed to determine the exact role of iron on the phenotypic and genotypic activity of *T. vaginalis*.

Keywords: *Trichomonas vaginalis*, metronidazole resistance, Iron

Introduction

The drug metronidazole and its related 5-nitroimidazole derivatives are effective against infections involving microaerophilic and anaerobic protozoa (1). These drugs have a selective antimicrobial activity that is reliant on the metabolic activation of the drug within the target cell, subsequently causing the release of reactive nitro anion radicals (2). Microorganisms that are susceptible to metronidazole possess electron generating and transport systems of low redox potential, that are capable of mediating the reduction of the drug (3). In *Trichomonas vaginalis*, a parasitic protozoan and the causative agent of the most common non-viral sexually transmitted infection (STI) in the world; trichomoniasis, the activation of metronidazole occurs in the organellar compartment called the hydrogenosome (4). *T. vaginalis* colonizes the urogenital tract of both men and woman (5). Although in men the spontaneous resolution of the sexually transmitted disease is common, in women the infection can persist for long periods and result in HIV infection, adverse pregnancy outcomes and furthermore, neoplasia's of the cervix (6-7). Despite having high carbon dioxide (CO_2) requirements, *T. vaginalis* is not strictly anaerobic as it consumes oxygen (O_2) in low amounts, which correlates with the microaerophilic environment that exists in the vaginal cavity (8).

The key hydrogenosomal enzyme, *pyruvate ferredoxin oxidoreductase (PFOR)*, generates electrons that are essential for metronidazole reduction, and catalyses the decarboxylation of *pyruvate* to *acetyl-CoA* and CO_2 (9). *Adenosine triphosphate (ATP)* production paired with the above reaction at substrate level produces a final product of acetate (10-12). The electrons released in the *PFOR* reaction are accepted by *ferredoxin* that can then be re-oxidized by hydrogenase (13). Another final product of this reaction is molecular hydrogen, which is produced due to the reaction electrons pairing with protons (14). Metronidazole acts as a superior electron acceptor when it is present, thus competing with hydrogenase for electrons (15). Effectively, the activation of metronidazole can be reflected in the reduced hydrogen production by the hydrogenosomes (!5).

Metronidazole resistance in *T. vaginalis* can be divided into anaerobic resistance and aerobic resistance (16). Anaerobic resistance has been associated with a down-regulation of the metronidazole-activating enzymes, *PFOR* and *ferredoxin* (17-18). Contrastingly, aerobic resistance to metronidazole is caused due to a decrease in the oxygen scavenging capacity that results in higher oxygen concentrations inside the hydrogenosome (19). The oxygen is able to re-oxidise the nitro-radical anions, thus decreasing their effect. *Flavin reductase*, another enzyme, is part of the oxygen defence system in *T. vaginalis* and is not expressed in highly resistant *T. vaginalis* (20). A restriction of iron in the nutritional requirements of *T. vaginalis* has been identified to reduce the metabolic activity of the hydrogenosome, and down-regulate the genes encoding the hydrogenosomal proteins (21-22). While previous studies have elucidated the role of an iron-independent mechanism of metronidazole activation through *flavin reductase*, *T. vaginalis* depends on *flavin reductase* for iron metabolism (23). Therefore, *T. vaginalis* that are resistant to metronidazole are forced to alter their iron-dependant pathways if this enzyme is deficient (2). Noticeably, it has been described that iron accessibility influences hydrogenosomal catabolism and a constricted iron nutritional environment can result in the decreased metabolic activity of hydrogenosomes, subsequently down-regulating the genes encoding for hydrogenosomal proteins (24). Studies have also shown that *T. vaginalis* can hinder and restrict metronidazole activation due to numerous down-regulated genes occurring in resistant isolates and alterations in iron-dependent processes (25). This study assessed the role of iron on the regulation of genes associated with metronidazole resistance in *T. vaginalis* isolates from South Africa. This study also investigated the association between iron supplementation and metronidazole susceptibility patterns in the *T. vaginalis* isolates.

Methods

Culturing of T. vaginalis isolate isolates

Eleven stored isolates of *T. vaginalis* were used in this study (n=2 resistant isolates, n=8 intermediate isolates, n=1 susceptible isolate). The isolates were cultured from self-collected vaginal swabs

obtained from antenatal women attending care at the King Edward VIII in Durban, South Africa. The stored isolates were grown in tubes containing diamonds TYM media supplemented with antibiotics (ampicillin, amphotericin B, ciprofloxacin, chloramphenicol, vancomycin and amikacin), to prevent the growth of unwanted microbes. The isolates were incubated for three to five days at 37°C and were routinely checked for optimal cell growth using wet mount microscopy. Isolates were considered optimally grown if cells with a jerk motility were observed.

Metronidazole susceptibility assays in the presence and absence of iron

Metronidazole susceptibility assays were performed in the presence of iron using media containing different increased concentrations of $FeSO_4$ (0 μM, 30 μM, 60 μM, and 120 μM). Susceptibility of trichomonads to metronidazole was determined *in vitro*. Briefly, 1.5 x 10^5 *T. vaginalis* trophozoites per ml were incubated in the presence of serially diluted metronidazole (0–20 ug/ml for susceptible strains and 0–200 ug/ml for resistant strains) in the TYM medium (1.0 ml final volume), using microtubes at 37°C. Isolates were also grown up with no $FeSO_4$. This served as a control.

The viability of the cells was determined by the trypan blue exclusion assay. Parasites were counted using a hemocytometer. Minimum inhibitory concentrations (MICs) of metronidazole, at which non-viable cells were present, were considered as the metronidazole MIC. The experiment was repeated at least three times to confirm the MIC results.

RNA Extraction

Total RNA was extracted from the cell pellets using the PureLink RNA Mini Kit (ThermoFisher Scientific, United States), according to the manufacturer's instructions. Cells were transferred to a 15 ml tube and centrifuged at 2,000 x g for 5 minutes at 4°C to pellet the cells. The kit instructions were then followed without any modifications. The purified RNA was then stored on ice and converted immediately to cDNA.

Synthesis of complementary DNA

The extracted RNA (7µl) was converted to cDNA using the Maxima H Minus cDNA synthesis Master Mix with *dsDNase* (ThermoFisher Scientific, United States) according to the manufacturer's instructions. A nanodrop was used to measure the concentration (ng/µl) of the cDNA. The converted cDNA was stored at -20°C until further use.

Regulation of genes associated with metronidazole resistance in the presence and absence of iron

The fold change in expression of target genes was assessed using the PowerUP SYBR Green Master Mix (ThermoFisher Scientific, United States). The sequences of the PCR primers are shown in Table 1. A 10 µl reaction was prepared using 0.3 µl of forward and reverse primers, 1 µl of DNA, 5 µl of PowerUP and 3.4 µl of water. PCR amplification was performed on the Quant Studio 5 real-time PCR detection system (ThermoFisher Scientific, United States), in a 96-well microtiter reaction plate. The cycling conditions comprised of a (Uracil-DNA Glycosylase) UDG activation stage for 2 minutes at 50°C, initial denaturation for 2 minutes at 95°C, followed by 40 cycles consisting of denaturation for 15 seconds at 95°C, annealing 15 seconds at 60°C with extension for 1 min at 72°C. To assess any contamination in the PCR, a negative control was included in all PCR runs. Detection of amplified fluorescent products was carried out at the end of the extension phase. The raw fluorescent data that included the Ct mean values were automatically generated by the Quant Studio 5 Real-time PCR system software. The experiment included an endogenous control (β-tubulin) and, a negative control which had no DNA template. The formula used to calculate the expression fold exchange was $\Delta\Delta C_T = \Delta C_{T\text{treated sample}} - \Delta C_{T\text{reference sample}}$.

Table 1. The sequences of the PCR primers used for the expression analysis

	Primer Sequence	(5'-3')
Genes	**Forward**	**Reverse**
Flavin reductase 1	CTTGATGTCTCACATGCACG	TTGGCTGAATCAGCGAAACG
PFOR	CTGCAAGCTCCTTACACAGC	AAGAGGGAGTTAGCCCAAGC
Ferredoxin	TGCCGCTTTGGAACAATCA	TGTCTGGTCATCTTCGAACTTGA
β-Tubulin	AAGATGGGTGTTTTAAGCTAGATAAGT	CGTCTTCAAGTATGCCCCAGTAC
ntr4	GTCAGAGGCCAAGAAAAGCTTGCC	GAGCATCGCAGGTGATAACGTTC
ntr6	CGTTGTTACAAACAAGGAAAAACTCC	GGATGCACGCTCATTCTTGA

Statistical analyses

Statistical analyses were conducted with GraphPad Prism 6.0 (GraphPad, San Diego, United States). Statistically significant expression changes had been calculated using one-way ANOVA. The level of significance was also determined by the Bonferroni method comparing all the test groups to the control group ($p< 0.05$).

Results

Effects of iron on the growth of T. vaginalis

According to Figure 1, FeSO4 concentrations between 20 to 40uM, did not affect the growth of the *T. vaginalis* isolates, however at concentrations above 40uM, a decline in the growth of *T. vaginalis* isolates was observed. An iron concentration of 120uM resulted in the death of all viable trophozoites.

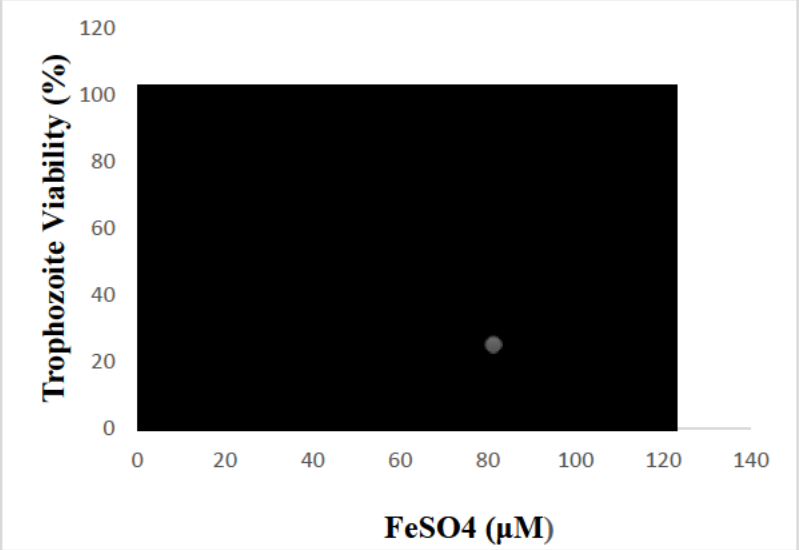


Figure 1: The growth curve of *T. vaginalis* isolates in the presence of varying iron concentrations. The MIC and viability of trophozoites was assessed in the presence of 0 µM, 30 µM, 60 µM, and 120 µM of ferrous sulphate ($FeSO_4$). The isolates were grown up in a normal medium without any metronidazole. The plot on the graph represents the mean with standard deviations of eleven independent data sets.

Susceptibility assays to metronidazole in the presence and absence of iron

In the absence of iron, 9.09% (1/11) of the isolates were susceptible to metronidazole, 72.7% (8/11) had an intermediate susceptibility profile and 18.2% (2/11) were resistant to metronidazole (Table 3). However, in the presence of 30 µM of $FeSO_4$, 45.5% (5/11) of the isolates had an intermediate susceptibility profile whilst 54.5% (6/11) were susceptible to metronidazole. None of the isolates exhibited a resistance profile. In the presence of 60 µM of $FeSO_4$, 18% (2/11) of the isolates had an intermediate susceptibility profile whereas 81.8% (9/11) were susceptible to metronidazole (Table 2). Supplementation with 120 µM of $FeSO_4$ was not performed for the metronidazole susceptibility assays since this concentration of iron resulted in complete death of trophozoites as shown in Figure 1.

Table 2: Metronidazole susceptibility data in the presence and absence of iron

T. vaginalis isolates	Metronidazole MIC in absence of Iron (µg/ml)	Susceptibility Profile in absence of Iron	Iron Concentration (µM)	Metronidazole MIC in presence of Iron (µg/ml)	Susceptibility Profile in presence of Iron
TV101	2	Intermediate	30	2	Intermediate
			60	1	Susceptible
TV182	2	Intermediate	30	1	Susceptible
			60	1	Susceptible
TV179	1	Susceptible	30	1	Susceptible
			60	1	Susceptible
TV209	2	Intermediate	30	1	Susceptible
			60	1	Susceptible
TV211	2	Intermediate	30	2	Intermediate
			60	1	Susceptible
TV230	2	Intermediate	30	0.5	Susceptible
			60	0.5	Susceptible
TV233	2	Intermediate	30	0.5	Susceptible
			60	0.5	Susceptible
TV241	2	Intermediate	30	1	Susceptible
			60	1	Susceptible
TV253	4	Resistant	30	2	Intermediate
			60	2	Intermediate
TV270	4	Resistant	30	2	Intermediate
			60	2	Intermediate
TV341	2	Intermediate	30	2	Intermediate
			60	1	Susceptible

Regulation of genes associated with metronidazole susceptibility patterns in the presence and absence of iron

For the gene expression assays, n=2 metronidazole resistant isolates (TVST-MR1 and TVST-MR2) and n=1 metronidazole susceptible isolate (TVST-MS1) were analysed. Since the amplification of the individual genes were undetermined in the susceptible isolate (TVST-MS), gene expression profiles were not generated for this isolate. The expression data for the individual genes is shown in Table 3.

Table 3: Expression data for the individual genes

Isolates grown without iron								
Endogenous control (β-tubulin)			**Gene of Interest (PFOR)**					
Sample	**Ct**	**Ct mean**	**Sample**	**Ct**	**Ct mean**	**Δ C_T Mean**	**ΔΔC_T**	**Fold difference $2^{-\Delta\Delta CT}$**
Reference sample (179)	0	0	Control 1 (179)	30.40	33.08	33.08	0.00	1.00
Reference sample (179)	0		Control 2 (179)	33.09				
Reference sample (179)	0		Control 3 (179)	35.75				
Treated 1 (253)	0	0	Treated 1 (253)	28.95	28.29	28.29	-4.79	27.72
Treated 2 (253)	0		Treated 2 (253)	28.48				
Treated 3 (253)	0		Treated 3 (253)	27.43				
Treated 1 (270)	0	0	Treated 1 (270)	27.81	27.85	27.85	-5.23	37.44
Treated 2 (270)	0		Treated 2 (270)	28.67				
Treated 3 (270)	0		Treated 3 (270)	27.08				

Isolates grown with iron								
Endogenous control (B-TUBULIN)			**Gene of Interest (PFOR)**					
Sample	**Ct**	**Ct mean**	**Sample**	**Ct**	**Ct mean**	**Δ C_T Mean**	**ΔΔC_T**	**Fold difference $2^{-\Delta\Delta CT}$**
Reference sample (179)	0	0	Control 1 (179)	28.34	28.75	28.75	0.00	1.00
Reference sample (179)	0		Control 2 (179)	28.01				
Reference sample (179)	0		Control 3 (179)	29.90				
Treated 1 (253)	0	0	Treated 1 (253)	25.57	23.61	23.61	-5.13	35.13
Treated 2 (253)	0		Treated 2 (253)	23.84				
Treated 3 (253)	0		Treated 3 (253)	21.43				
Treated 1 (270)	0	0	Treated 1 (270)	23.31	23.45	23.45	-5.30	39.51
Treated 2 (270)	0		Treated 2 (270)	23.82				
Treated 3 (270)	0		Treated 3 (270)	23.21				
Isolates grown with iron								
Endogenous control (β-tubulin)			**Gene of Interest (ferredoxin)**					
Sample	**Ct**	**Ct mean**	**Sample**	**Ct**	**Ct mean**	**Δ C_T Mean**	**ΔΔC_T**	**Fold difference $2^{-\Delta\Delta CT}$**
Reference sample (179)	0	0	Control 1 (179)	32.13	34.29	34.29	0.00	1.00
Reference sample (179)	0		Control 2 (179)	36.19				

Reference sample (179)	0		Control 3 (179)	34.55				
Treated 1 (253)	0	0	Treated 1 (253)	29.60	28.77	28.77	-5.52	45.83
Treated 2 (253)	0		Treated 2 (253)	28.58				
Treated 3 (253)	0		Treated 3 (253)	28.13				
Treated 1 (270)	0	0	Treated 1 (270)	29.34	30.24	30.24	-4.05	16.58
Treated 2 (270)	0		Treated 2 (270)	30.50				
Treated 3 (270)	0		Treated 3 (270)	30.87				
Isolates grown without iron								
Endogenous control (β-tubulin)			**Gene of Interest (ferredoxin)**					
Sample	**Ct**	**Ct mean**	**Sample**	**Ct**	**Ct mean**	**Δ C_T Mean**	**ΔΔC_T**	**Fold difference $2^{-\Delta\Delta CT}$**
Reference sample (179)	0	0	Control 1 (179)	30.68	29.17	29.17	0.00	1.00
Reference sample (179)	0		Control 2 (179)	29.05				
Reference sample (179)	0		Control 3 (179)	27.79				
Treated 1 (253)	0	0	Treated 1 (253)	25.32	24.30	24.30	-4.88	29.36
Treated 2 (253)	0		Treated 2 (253)	23.87				
Treated 3 (253)	0		Treated 3 (253)	23.69				
Treated 1 (270)	0	0	Treated 1 (270)	25.79	25.67	25.67	-3.50	11.32

Treated 2 (270)	0		Treated 2 (270)	26.68				
Treated 3 (270)	0		Treated 3 (270)	24.55				
Isolates grown with iron								
Endogenous control (β-tubulin)			**Gene of Interest (flavin)**					
Sample	**Ct**	**Ct mean**	**Sample**	**Ct**	**Ct mean**	**Δ C_T Mean**	**ΔΔC_T**	**Fold difference $2^{-\Delta\Delta CT}$**
Reference sample (179)	0	0	Control 1 (179)	32.79	34.40	34.40	0.00	1.00
Reference sample (179)	0		Control 2 (179)	36.58				
Reference sample (179)	0		Control 3 (179)	33.81				
Treated 1 (253)	0	0	Treated 1 (253)	30.64	29.12	29.12	-5.28	38.77
Treated 2 (253)	0		Treated 2 (253)	29.46				
Treated 3 (253)	0		Treated 3 (253)	27.26				
Treated 1 (270)	0	0	Treated 1 (270)	29.86	29.50	29.50	-4.89	29.66
Treated 2 (270)	0		Treated 2 (270)	29.84				
Treated 3 (270)	0		Treated 3 (270)	28.82				
Isolates grown without iron								
Endogenous control (β-tubulin)			**Gene of Interest (flavin)**					
Sample	**Ct**	**Ct mean**	**Sample**	**Ct**	**Ct mean**	**Δ C_T Mean**	**ΔΔC_T**	**Fold difference $2^{-\Delta\Delta CT}$**

Reference sample (179)	0	0	Control 1 (179)	29.01	27.97	27.97	0.00	1.00
Reference sample (179)	0		Control 2 (179)	27.04				
Reference sample (179)	0		Control 3 (179)	27.85				
Treated 1 (253)	0	0	Treated 1 (253)	24.64	24.12	24.12	-3.85	14.43
Treated 2 (253)	0		Treated 2 (253)	25.46				
Treated 3 (253)	0		Treated 3 (253)	22.26				
Treated 1 (270)	0	0	Treated 1 (270)	24.86	24.84	24.84	-3.13	8.76
Treated 2 (270)	0		Treated 2 (270)	26.84				
Treated 3 (270)	0		Treated 3 (270)	22.82				
Isolates grown with iron								
Endogenous control (β-tubulin)			**Gene of Interest (ntr4)**					
Sample	**Ct**	**Ct mean**	**Sample**	**Ct**	**Ct mean**	**Δ C_T Mean**	**ΔΔC_T**	**Fold difference $2^{-\Delta\Delta CT}$**
Reference sample (179)	0	0	Control 1 (179)	33.40	35.98	35.98	0.00	1.00
Reference sample (179)	0		Control 2 (179)	38.00				
Reference sample (179)	0		Control 3 (179)	36.55				
Treated 1 (253)	0	0	Treated 1 (253)	33.05	32.71	32.71	-3.28	9.69
Treated 2 (253)	0		Treated 2 (253)	32.63				

Treated 3 (253)	0		Treated 3 (253)	32.44				
Treated 1 (270)	0	0	Treated 1 (270)	34.17	33.98	33.98	-2.01	4.02
Treated 2 (270)	0		Treated 2 (270)	33.79				
Treated 3 (270)	0		Treated 3 (270)	33.97				
Isolates grown without iron								
Endogenous control (β-tubulin)			**Gene of Interest (ntr4)**					
Sample	**Ct**	**Ct mean**	**Sample**	**Ct**	**Ct mean**	**Δ C_T Mean**	**ΔΔC_T**	**Fold difference $2^{-\Delta\Delta CT}$**
Reference sample (179)	0	0	Control 1 (179)	30.80	28.52	28.52	0.00	1.00
Reference sample (179)	0		Control 2 (179)	26.25				
Reference sample (179)	0		Control 3 (179)	28.51				
Treated 1 (253)	0	0	Treated 1 (253)	27.44	27.49	27.49	-1.03	2.05
Treated 2 (253)	0		Treated 2 (253)	27.32				
Treated 3 (253)	0		Treated 3 (253)	27.70				
Treated 1 (270)	0	0	Treated 1 (270)	26.88	26.80	26.80	-1.72	3.30
Treated 2 (270)	0		Treated 2 (270)	26.85				
Treated 3 (270)	0		Treated 3 (270)	26.67				
Isolates grown with iron								
Endogenous control (β-tubulin)			**Gene of Interest (ntr6)**					

Sample	**Ct**	**Ct mean**	**Sample**	**Ct**	**Ct mean**	**Δ C_T Mean**	**ΔΔC_T**	**Fold difference $2^{-\Delta\Delta CT}$**
Reference sample (179)	0	0	Control 1 (179)	34.50	34.04	34.04	0.00	1.00
Reference sample (179)	0		Control 2 (179)	33.68				
Reference sample (179)	0		Control 3 (179)	33.94				
Treated 1 (253)	0	0	Treated 1 (253)	28.54	28.48	28.48	-5.56	47.13
Treated 2 (253)	0		Treated 2 (253)	28.55				
Treated 3 (253)	0		Treated 3 (253)	28.36				
Treated 1 (270)	0	0	Treated 1 (270)	28.45	28.22	28.22	-5.82	56.56
Treated 2 (270)	0		Treated 2 (270)	28.19				
Treated 3 (270)	0		Treated 3 (270)	28.02				
Isolates grown without iron								
Endogenous control (β-tubulin)			**Gene of Interest (ntr6)**					
Sample	**Ct**	**Ct mean**	**Sample**	**Ct**	**Ct mean**	**Δ C_T Mean**	**ΔΔC_T**	**Fold difference $2^{-\Delta\Delta CT}$**
Reference sample (179)	0	0	Control 1 (179)	29.86	28.25	28.25	0.00	1.00
Reference sample (179)	0		Control 2 (179)	27.01				
Reference sample (179)	0		Control 3 (179)	27.90				
Treated 1 (253)	0	0	Treated 1 (253)	25.01	24.83	24.83	-3.43	10.74

Treated 2 (253)	0		Treated 2 (253)	24.70				
Treated 3 (253)	0		Treated 3 (253)	24.78				
Treated 1 (270)	0	0	Treated 1 (270)	24.75	24.73	24.73	-3.53	11.54
Treated 2 (270)	0		Treated 2 (270)	24.60				
Treated 3 (270)	0		Treated 3 (270)	24.82				

PFOR/Ferredoxin/Flavin, *ntr*4, *ntr*6: target gene

β-tubulin: reference gene and endogenous control

Sample 179 (Susceptible strain of *T. vaginalis*): Reference sample

Sample 253 and sample 270 (Resistance strain of *T. vaginalis*): Treated sample

- : below the detection threshold limits of the real-time PCR system

C_T : threshold cycle for target amplification

$\Delta C_T = C_{T(\textbf{Target})} - C_{T(\textbf{Reference})}$ i.e. $C_{T(\textbf{Flavin})} - C_{T(\textbf{β-Tubulin})}$

ΔC_T Mean= Mean of ($C_{T(\textbf{Flavin})} - C_{T(\textbf{β-Tubulin})}$)

$\Delta\Delta C_T = \Delta C_{T\text{treated sample}} - \Delta C_{T\text{reference sample}}$

$2^{-\Delta\Delta CT}$: Expression fold-change of the target (*Flavin*) normalised to the endogenous reference (β-tubulin), and relative to the reference sample

Pyruvate ferredoxin oxidoreductase (PFOR)

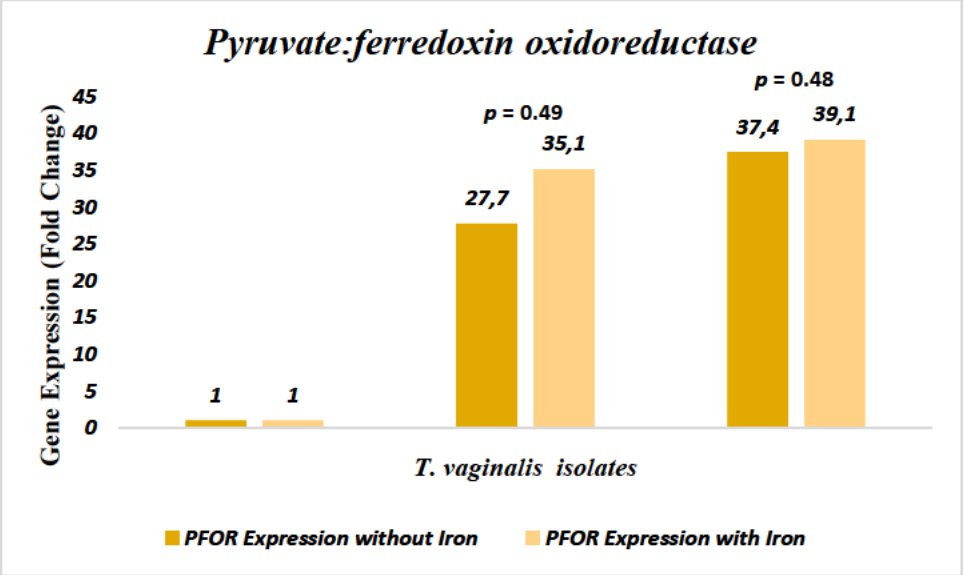


Figure 2: The regulation of the *pyruvate ferredoxin oxidoreductase (PFOR)* gene in the absence and presence of iron for metronidazole resistant isolates. The expression of the genes was higher in the presence of iron.

For isolate TVST-MR1, the expression of *PFOR* was higher in the presence of iron (35.1) when compared to expression in the absence of iron (27.7). There was no statistically significant difference in the expression of the gene in the presence and absence of iron, p=0.49. Similarly, for isolate TVST-MR2, the expression of *PFOR* was higher in the presence of iron (39.1) when compared to expression in the absence of iron (37.4). However, there was no statistically significant difference in the expression of the gene in the presence and absence of iron, p=0.48 (Figure 2). The expression levels across both resistant isolates were fairly similar, and therefore not statistically significant, p=0.33.

Flavin reductase 1

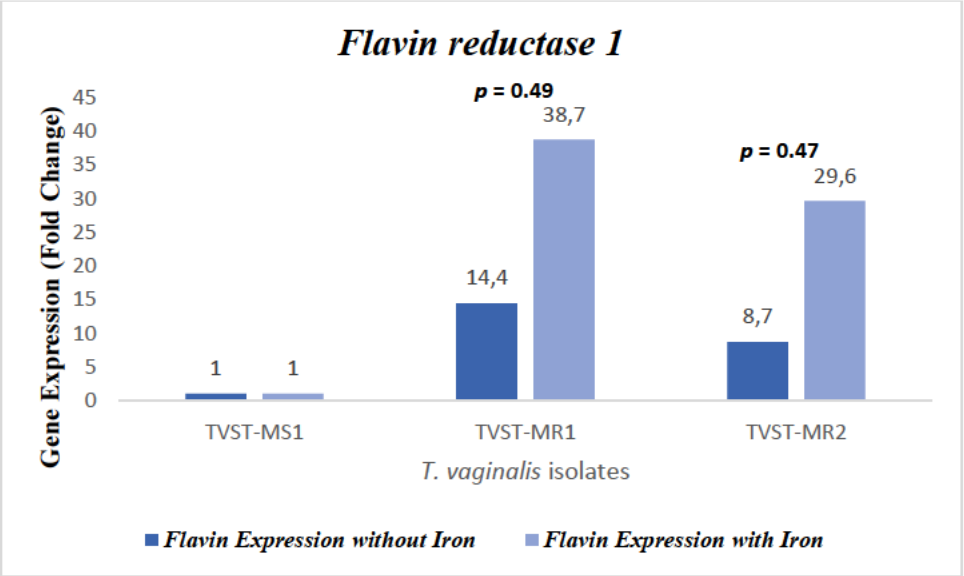


Figure 3: The regulation of the *flavin reductase 1* gene in the absence and presence of iron for metronidazole resistant isolates. The expression of the genes was higher in the presence of iron.

For isolate TVST-MR1, the expression of *flavin reductase 1* was slightly higher in the presence of iron (38.7) when compared to expression in the absence of iron (14.4). There was no statistically significant difference in the expression of the gene in the presence and absence of iron, p=0.49. For isolate TVST-MR2, the expression of *flavin reductase 1* was higher in the presence of iron (29.6) when compared to expression in the absence of iron (8.7). However, there was no statistically significant difference in the expression of the gene in the presence and absence of iron, p=0.47 (Figure 3). Overall, the expression of the gene was higher in TVST-MR1 (3-fold more) when compared to TVST-MR2, however, this was not statistically significant, p=0.66.

Ferredoxin

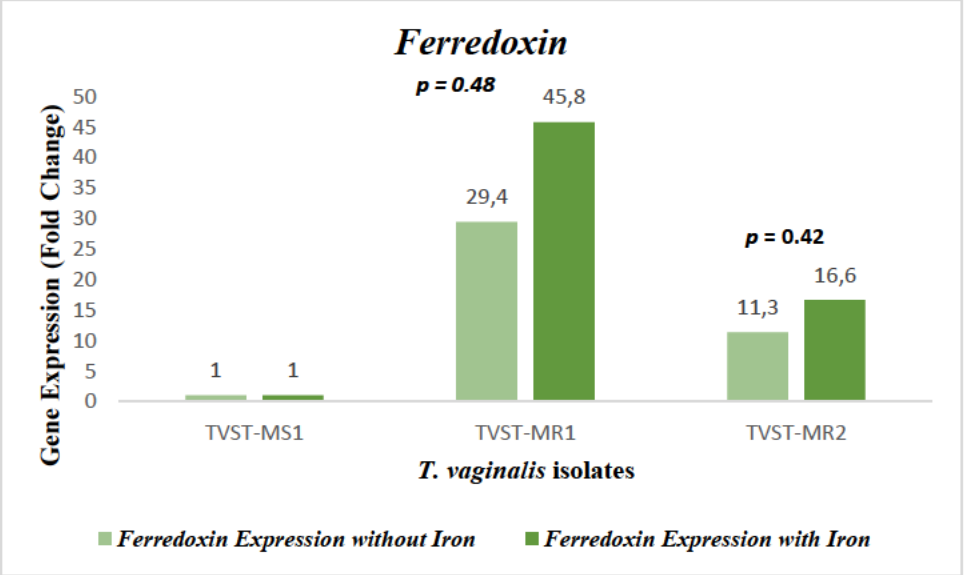
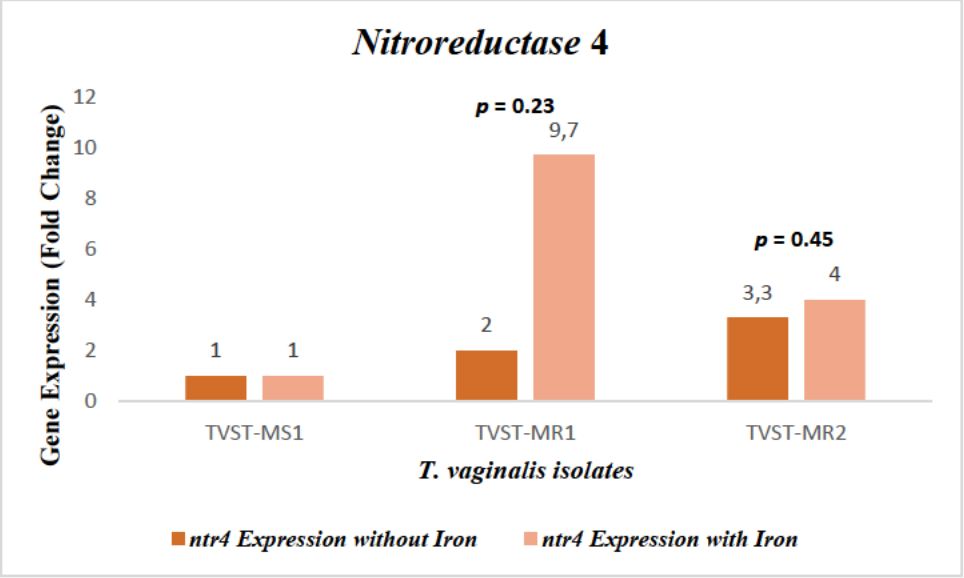


Figure 4: The regulation of the *ferredoxin* gene in the absence and presence of iron for metronidazole resistant isolates. The expression of the gene was higher in the presence of iron.

Overall, the expression of the *ferredoxin* gene was upregulated in the absence and presence of iron. For both resistant isolates, the expression levels were higher in the presence of iron (45.8 and 16.6) when compared to expression levels in the absence of iron (29.4 and 11.3). Despite the higher expression levels associated with the presence of iron, this was not statistically significant for both isolates, p=0.48 and p=0.42, respectively (Figure 4). When comparing the expression levels across the two isolates, overall, TVST-MR1 displayed higher expression levels when compared to TVST-MR2, however this was not statistically significant, p=0.99.

Nitroreductases



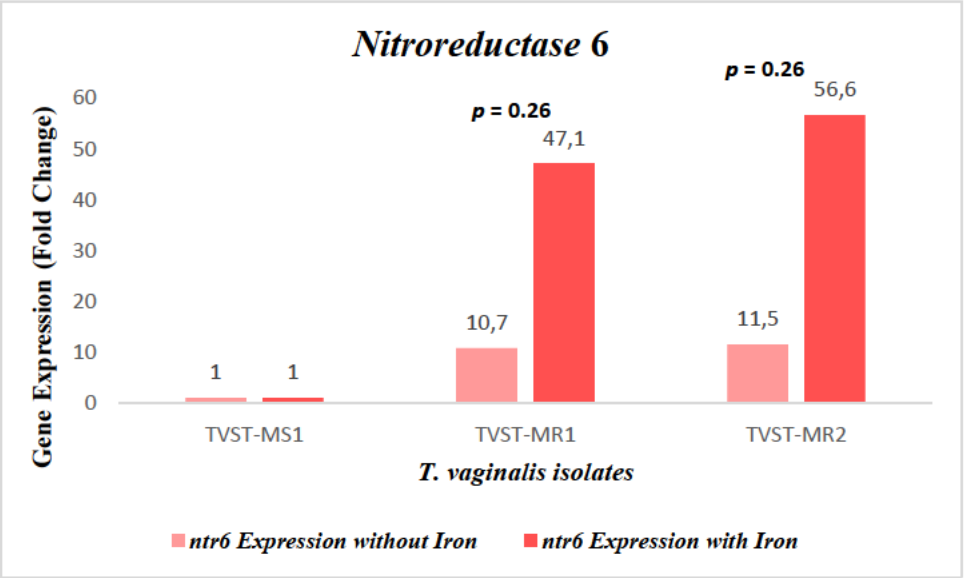


Figure 5: The regulation of the *nitroreductase 4* and *6* genes in the absence and presence of iron for metronidazole resistant isolates. The expression of the genes was higher in the presence of iron.

Overall, the expression of the *nitroreductase 4 and 6* genes were upregulated in the absence and presence of iron. For both resistant isolates, the expression of *nitroreductase 4* was higher in the presence of iron (9.7 and 4) when compared to expression levels in the absence of iron (2 and 3.3). Despite the higher expression levels associated with the presence of iron, this was not statistically

significant for both isolates, p=0.23 and p=0.45, respectively (Figure 5). When comparing the expression levels across the two isolates, overall, TVST-MR1 displayed higher expression levels in the presence of iron when compared to TVST-MR2, however this was not statistically significant, p=0.33.

With respect to *nitroreductase 6*, higher gene expression levels were observed when compared to *nitroreductase 4*. A similar trend to *nitroreductase 4* was observed, for both resistant isolates, the expression of *nitroreductase 6* was higher in the presence of iron (47.1 and 56.6) when compared to expression levels in the absence of iron (10.7 and 11.5). Despite the markedly higher expression levels associated with the presence of iron, this was not statistically significant for both isolates, p=0.26 and p=0.26, respectively (Figure 5). When comparing the expression levels across the two isolates, overall, TVST-MR2 displayed higher expression levels in the presence of iron when compared to TVST-MR1, however this was not statistically significant, p=0.33.

Discussion

In this study, the role of iron on the growth of *T. vaginalis* South African isolates was assessed. According to the analysis, increasing concentrations of iron in the absence of metronidazole resulted in a loss of viability of the trophozoites.

This suggests that iron may play a crucial role in the growth and survival of *T. vaginalis*. Previous studies have also investigated the effects of iron on *T. vaginalis* growth and survival. One study found that iron chelation led to a decrease in parasite viability and replication, indicating that iron acquisition is essential for *T. vaginalis* survival (26). Another study showed that *T. vaginalis* requires iron for the expression of key virulence factors, such as cysteine proteases (20). Iron is considered to be an essential element for the growth of many pathogens including *T. vaginalis*. Iron is not readily available *in vivo* and therefore *T. vaginalis* uses a multiplicity of mechanisms to obtain iron from their host, thus underscoring the importance of iron for microbial survival. Lehker and Alderete (1992) showed that

the withdrawal of iron from *T. vaginalis* cultures resulted in inhibited growth and multiplication of *T. vaginalis* due to a decreased rate of protein synthesis (28). These findings differ with the results obtained in the current study. The negative impact on the growth of *T. vaginalis* observed in our study may be due to iron-induced oxidative stress or the inhibition of key metabolic pathways, both of which have been suggested to occur in response to excess iron (27).

Iron is also vital for metabolic processes and the transcriptional regulation of genes responsible for key enzymes in *T. vaginalis* (27). The current study also assessed the role of iron supplementation on metronidazole susceptibility patterns. According to the analysis, the supplementation with iron to the susceptibility assays rendered the trophozoites more susceptible to metronidazole. Restriction of iron in *T. vaginalis* not only leads to the down-regulation of genes important for energy metabolism, but it also downregulates those genes needed for drug activation, such as *PFOR* and *ferredoxin*. Although iron is important for *T. vaginalis* growth, excess iron contributes to lowering the MICs of metronidazole. In this study, the addition of iron to the drug susceptibility assay media resulted in an increased susceptibility to metronidazole. Similar observations have been reported by Horvathova *et al*. 2012 (29), in that the addition of iron to metronidazole treatment increased sensitivity to metronidazole. Kulda et al. 1993 reported that the down-regulation of genes such as *ferredoxin*, *flavin reductase* and *PFOR* was associated with metronidazole resistance (31). In this study, the genes *ferredoxin*, *flavin reductase*, *PFOR* and *nitroreductases 4* and *6* were not expressed in the metronidazole susceptible isolate, suggesting that they are only expressed in metronidazole resistant isolates, which have been observed in this study.

Previous studies have suggested that iron may plays a role in the regulation of *flavin* biosynthesis in *T. vaginalis*. However, in the current study, that there was no significant difference in the expression of *flavin* in the presence or absence of iron. Previous studies have shown that the upregulation of *flavin* biosynthesis genes is a key mechanism for metronidazole resistance in *T. vaginalis*. A past study found that the expression of *flavin* biosynthesis genes was significantly higher in metronidazole-resistant

strains of *T. vaginalis* when compared to susceptible isolates (32). Similarly, in the current study, the *flavin* gene was only expressed in the resistant isolates and not in the susceptible isolate.

Past studies have shown that the upregulation of energy metabolism pathways, including *PFOR*, is a key mechanism for metronidazole resistance in *T. vaginalis* (31). The findings of the current study are consistent with previous studies that have shown that the upregulation of energy metabolism pathways, including *PFOR*, is a key mechanism of metronidazole resistance in *T. vaginalis* since this gene was only expressed in the *T. vaginalis* resistant isolates and not the susceptible isolate. Although the expression of *PFOR* was higher in the presence of iron, there was no significant association between the presence or absence of iron and expression of the gene. A previous study found that the expression of genes involved in energy metabolism was significantly higher in metronidazole-resistant strains of *T. vaginalis* compared to susceptible strains (40). The study also found that treatment with the *PFOR* inhibitor benzimidazole-2-yl-carbamate led to a decrease in *PFOR* activity and an increase in metronidazole sensitivity (41).

Ferredoxin is a protein that plays a critical role in the electron transfer chain of anaerobic organisms, including *T. vaginalis* (31). The results show that the expression of the *ferredoxin* gene is upregulated in metronidazole-resistant isolates in both the absence and presence of iron compared to the metronidazole-susceptible isolate. This indicates that the resistant isolates are more metabolically active and are better able to survive metronidazole treatment when compared to the susceptible isolate.

The upregulation of the *ferredoxin* gene has been previously associated with metronidazole resistance in *T. vaginalis*. A study by Huang *et al*. (2017) found that the expression of the *ferredoxin* gene was significantly upregulated in metronidazole-resistant isolates when compared to susceptible isolates (34). Furthermore, the study also found that the addition of iron in the culture media further increased the expression of the *ferredoxin* gene in the metronidazole-resistant isolates, as seen in the present

results. This suggests that the availability of iron may contribute to metronidazole resistance in *T. vaginalis*.

Although both aerobic and anaerobic pathways of metronidazole resistance have been proposed, *T. vaginalis* was grown under microaerophilic conditions in this study. Anaerobically resistant isolates of *T. vaginalis* are identified by the absence of certain hydrogenosomal metabolic enzymes that usually activate metronidazole (34). Although past studies have investigated the role of these genes in metronidazole resistance, alterations in these genes do not explain all cases of metronidazole resistance (31, 36-39). Additional factors modulating metronidazole resistance in *T. vaginalis* also exist, specifically the *nitroreductases* (NTR) (40).

NTR, an enzyme responsible for the reduction of 5-nitroimidazoles, have been identified to have single nucleotide polymorphisms in two *nitroreductase* genes (*ntr4* and ntr6) that are associated with metronidazole resistance (40). In this study, the *ntr4* and *ntr6* carrying the drug resistance mutations were only expressed in the resistant isolates. The expression of *ntr4* and *ntr6* was higher in the presence of iron, however, there was no significant association between the presence or absence of iron and expression of the genes.

Conclusion

In this study, high concentrations of iron resulted in total loss of viability of *T. vaginalis* isolates. At lower iron concentrations, iron supplementation increased the antimicrobial activity of metronidazole on *T. vaginalis* isolates by switching from resistant to susceptible phenotypes. In addition, the presence of iron resulted in an increase in the expression of resistance genes, but as expected this was not significant due to the antimicrobial properties of iron on the growth of *T. vaginalis* as shown in this study. Future studies are needed to determine the exact role of iron on the phenotypic and genotypic activity of *T. vaginalis*.

Limitations

A small sample size (11) was available for testing. Of this, only two isolates were resistant. In addition, although study participants were recruited from a single clinic, this clinic in King Edward VIII hospital is a central tertiary hospital that services most of Durban's population. Future research should look at investigating additional potential genes associated with metronidazole resistance in a larger sample of isolates. The use of iron in future antimicrobials should be investigated to aid in the inhibition of *T. vaginalis*. This is being considered as a future research endeavour.

Conflicts of Interest

The authors declare no conflict of interest.

Funding: National Research Foundation, College of Health Sciences funding (University of KwaZulu-Natal)

Ethical Standards

Ethical approval was obtained from the Biomedical Research Ethic Committee (BREC) (BREC/00003557/2021).

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CHAPTER FOUR

This manuscript was submitted to Infectious Diseases in Obstetrics and Gynecology (Submission ID 1962038)

Synergistic Strategies: Harnessing Plant Nanoemulsions to Combat Metronidazole-Resistant *Trichomonas vaginalis* in South Africa

Rowen Govender[a,*] Deshanta Naicker[a], Swenkile Ndlovu[b] and Nathlee S Abbai[a]

[a]Department of Clinical Medicine, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa

[b]Department of Medical Microbiology, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa

[*]***Corresponding author:***

Rowen Govender

Department of Clinical Medicine,

College of Health Sciences,

Nelson R. Mandela School of Medicine,

University of KwaZulu-Natal,

Durban, South Africa

Email: rowengovende@gmail.com; ABBAIN@ukzn.ac.za

ORCID Number: 0000-0003-4820-4574

Abstract

Trichomoniasis, caused by the protozoan parasite *Trichomonas vaginalis*, is a prevalent sexually transmitted infection with significant public health implications, particularly in regions where metronidazole resistance has emerged as a critical concern. In light of this challenge, alternative and innovative treatment approaches are urgently needed. This study explored the potential of plant-based nanoemulsions as a novel therapeutic strategy against metronidazole-resistant *T. vaginalis* isolates.

Three medicinal plants, *Ocimum tenuiflorum, Moringa oleifera*, and *Azadirachta indica*, known for their antimicrobial properties were selected as sources of bioactive compounds. Nanoemulsions were prepared to enhance the solubility and bioavailability of these plant extracts. Our findings revealed a concentration-dependent response on the susceptibility of *T. vaginalis* isolates to these nanoemulsions. Notably, nanoemulsion introduction led to a shift from resistance to susceptibility in metronidazole-resistant isolates, with minimum inhibitory concentrations (MIC) values decreasing from 4 µg/ml to 1 µg/ml. This observation underscores the potential of nanoemulsions to restore the efficacy of plant-based treatments against metronidazole-resistant isolates.

Furthermore, the study unravelled intriguing concentration-dependent effects of the plant extracts on the viability of vaginal epithelial cells. *Azadirachta indica* and *Moringa oleifera* extracts exhibited hormetic responses, with low concentrations enhancing cell viability, while higher concentrations exhibited cytotoxicity. Conversely, *Ocimum tenuiflorum* extract displayed a protective effect at moderate concentrations. These complex interactions between plant extracts and host cells highlight the need for further mechanistic investigations.

In conclusion, our research provides promising evidence of the potential of plant-based nanoemulsions as alternative treatments for metronidazole-resistant trichomoniasis. The adaptability of these treatments to diverse healthcare settings, combined with their affordability and accessibility, offers hope in the battle against this challenging public health issue. Future studies should investigate the

underlying mechanisms of action and consider *in vivo* evaluations to translate these findings into effective clinical therapies.

Key words: *Trichomonas vaginalis*, nanoemulsions, metronidazole resistance

Introduction

Trichomoniasis, a sexually transmitted infection caused by the protozoan parasite *Trichomonas vaginalis*, stands as a formidable and escalating public health challenge that casts its shadow across numerous regions worldwide (Kissinger, 2015). South Africa, much like many other geographical areas, confronts the daunting task of managing this pervasive infection (Francis *et al.,* 2018). The gravity of the situation becomes increasingly evident when we consider the sheer prevalence of trichomoniasis cases and its impact on the overall health of the population.

In South Africa, a nation grappling with a high burden of infectious diseases, trichomoniasis takes on particular significance (Mabaso and Abbai, 2021). The coexistence of trichomoniasis with other prevalent infections, such as Human immunodeficiency virus/Acquired immunodeficiency syndrome (HIV/AIDS) and tuberculosis, compounds the challenges faced by the healthcare system (de Waai *et al.,* 2017). Individuals infected with trichomoniasis may be more susceptible to contracting HIV, and the presence of both infections can lead to more severe health consequences (de Waaij *et al.,* 2017). Therefore, the treatment and reduction in trichomoniasis becomes not only a matter of individual well-being but also a vital component of broader public health initiatives aimed at reducing the incidence and impact of infectious diseases in the region.

Metronidazole, a tried-and-true therapeutic agent for trichomoniasis, has historically been the mainstay of treatment, not only in South Africa but also globally (Bouchemal, Bories and Loiseaub, 2017). Its mechanism of action, based on the disruption of the DNA structure of *T. vaginalis*, has proven effective in curbing the spread of this parasitic infection (Bouchemal, Bories and Loiseaub, 2017). However, the emergence of metronidazole-resistant strains of *T. vaginalis* has cast a long shadow over the

effectiveness of this antibiotic (Meri *et al.,* 2000). This development underscores the dynamic nature of infectious diseases and the ever-evolving challenge of combating them.

In this intricate healthcare scenario, the exploration of alternative therapeutic avenues takes on a mantle of paramount importance. Medicinal plants, celebrated for their rich and diverse assortment of bioactive compounds, beckon as repositories of novel antimicrobial agents (Vaou *et al.,* 2021). These compounds, displaying remarkable versatility, offer tantalizing potential in targeting various facets of *T. vaginalis* biology, thereby holding out the promise of surmounting the challenge posed by metronidazole resistance (Mickymaray, 2019). Moreover, the use of locally available medicinal plants aligns with the principles of sustainability and traditional medicine, which have deep cultural roots in South Africa (Ndhlovu *et al.,* 2022).

However, their practical application in South Africa comes replete with formidable challenges, encompassing issues of bioavailability, stability, and controlled delivery (Sobiecki, 2014). The healthcare landscape of South Africa is marked by disparities in access to healthcare services, both in urban and rural areas. Therefore, any alternative treatment modality, including those derived from medicinal plants, must be not only efficacious but also affordable, accessible, and adaptable to the diverse healthcare settings within the country (Morris-Paxton, Reid an Ewing, 2020).

This pioneering study embarks on an ambitious mission to unravel the latent potential of nanoemulsions meticulously formulated from three specific plant sources—*Ocimum tenuiflorum* (holy basil) (Mallikarjun, 2016), *Azadirachta indica* (neem) (Wylie and Merrell, 2022), and *Moringa oleifera* (drumstick tree) (Jahan *et al.,* 2022) as bona fide alternatives for metronidazole-resistant strains of *T. vaginalis*. Through a rigorous scientific inquiry underpinned by experimentation, we aspire not only to contribute significantly to the evolving arsenal of locally pertinent treatment strategies but also to address a salient problem that looms large within South Africa's healthcare domain: the escalating resistance to metronidazole in treating trichomoniasis (Ndhlovu *et al.,* 2022).

The weightiness of this research endeavour assumes even greater gravity when considered within the broader context of South Africa's healthcare landscape. The consequences of untreated or inadequately managed trichomoniasis reverberate across various dimensions, encompassing individual health, public health systems, and socioeconomic realms (Mabaso and Abbai, 2021). The quest for alternatives to metronidazole, therefore, extends beyond a mere scientific exploration; it represents a pressing public health imperative with far-reaching implications.

Methods

Collection and Preparation of the plant materials

Plant Selection: The three plant sources, namely *Ocimum tenuiflorum, Azadirachta indica*, and *Moringa oleifera*, were selected based on their established antimicrobial properties and traditional medicinal uses.

Plant collection and preparation of extracts

The leaves of the three-plant species, namely *Ocimum tenuiflorum*, *Moringa oleifera*, and *Azadirachta indica*, were gathered from the Botanical Gardens located in Durban, South Africa (29.8483° S, 31.0083° E). Upon collection, the leaves were meticulously cleaned to eliminate any dust and dirt. To ensure cleanliness, the leaves were first immersed in deionized water for one minute. Subsequently, the leaves were allowed to naturally air dry for a duration of 4 days (Figure 1). During this drying period, precautions were taken to shield the leaves from direct sunlight.

Figure 1: Preparation of plant materials *Ocimum tenuiflorum* (A), *Moringa oleifera* (B) and *Azadirachta indica* (C).

For the extraction of plant compounds, the dried leaves were carefully cut into small pieces using sterilized scissors. Approximately 100 grams of each leaf type was combined with 500 ml of distilled water and subjected to boiling at 100°C for a duration of 30 minutes (Figure 2A). After the boiling process, the mixture was allowed to cool down and then filtered to obtain the desired aqueous extract, as depicted in Figure 2. Further refinement of the extracts involved additional filtration steps and centrifugation to eliminate any residual solid particles. These resulting extracts were stored at a temperature of 20°C until they were ready for use in the study.

Nanoemulsion Preparation

For the creation of the oil phase, we initiated the process by generating a homogeneous organic solution, denoted as S1. This solution consisted of 400 µl of isopropyl myristate and 86 µl of span 80, a lipophilic surfactant, dissolved in a solvent compatible with water (Figure 2 B and C).

In parallel, the aqueous phase was prepared by forming another homogeneous solution, referred to as S2, which involved mixing 80 ml of water with 136 µl of Tween 80, a hydrophilic surfactant. While subjecting this aqueous phase to magnetic stirring, we introduced 30 ml of the plant extract. This addition promptly led to the formation of an oil-in-water (o/w) emulsion, as the organic solvent

diffused into the surrounding aqueous medium, resulting in the formation of nano-sized droplets. Magnetic stirring was continued for a duration of 30 minutes to allow the system to reach equilibrium.

To eliminate any traces of the water-miscible solvent, the emulsion was exposed to evaporation under reduced pressure for 45 minutes, accompanied by centrifugation at 1000 rpm. Subsequently, the emulsion was rapidly cooled by immersing it in an ice bath for a period of 10 minutes (as illustrated in Figure 2). Nanoemulsion concentrations of 1000 µM were meticulously prepared and stored at a temperature of 4°C (Figure 2D).

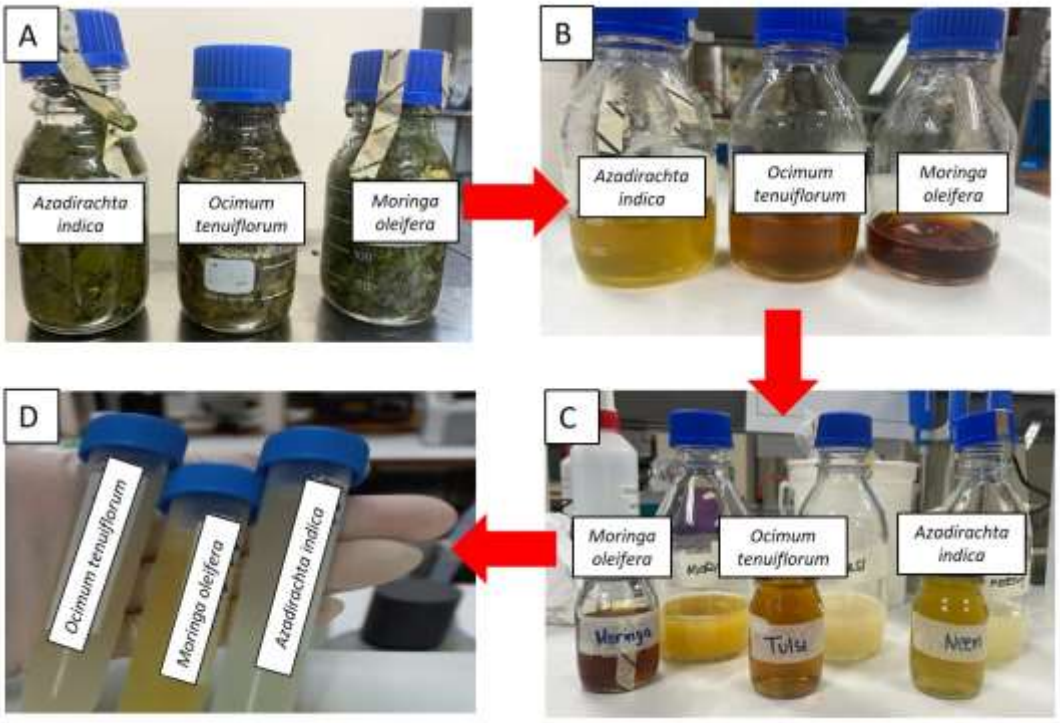


Figure 2: Nanoemulsion preparation from plant extracts.

Culturing of Trichomonas vaginalis

Five stored *T. vaginalis* isolates were used in this study (n=2 resistant isolates, n=2 susceptible isolate, n=ATCC control). The isolates were cultured from self-collected vaginal swabs obtained from antenatal women attending care at the King Edward VIII in Durban, South Africa. The stored isolates were grown in tubes containing diamonds TYM media supplemented with antibiotics (ampicillin,

amphotericin B, ciprofloxacin, chloramphenicol, vancomycin and amikacin), to prevent the growth of unwanted microbes. The isolates were incubated for three to five days at 37°C with a controlled atmosphere of 5% CO_2 and were routinely checked for optimal cell growth using wet mount microscopy. Sub-culturing was performed regularly to ensure the viability of the cultures. Isolates were considered optimally grown if cells with a jerk motility were observed.

Nanoemulsion susceptibility assays

Nanoemulsion susceptibility of each plant was performed in 96 well flat-bottomed microtiter plates under anaerobic incubation conditions. Two-fold serial dilutions of each nanoemulsion was performed in Diamond's TYM medium. The resulting concentrations ranged from 0.25 µg/ml to 16 µg/ml. *T. vaginalis* cultures were then standardized to an inoculum of $1.5x10^4$ trichomonads/well. Each *T. vaginalis* isolate inoculum was then added into each well excluding the ATCC control wells. The *T. vaginalis* ATCC 50148 strain was used as a control strain and untreated cultures of the respective isolates were used as growth controls.

Plates were incubated in air-tight anaerobic jars containing Oxoid™ AnaeroGen™ 2.5L gas packs (ThermoFisher Scientific, United States) and Oxoid™ Resazurin Anaerobic indicator strips (ThermoFisher Scientific, United States) at 37ºC for 48 hours. *T. vaginalis* motility and growth was assessed using the inverted microscope at × 400 magnification and scored according to the scoring criteria described by Upcroft (Upcroft and Upcroft 2001). Trophozoite numbers were scored 1+ (0-10 motile parasites; not more than 20% coverage of well surface and significantly less active), 2+ (20 to 50% coverage of the well surface and some trophozoite motility), 3+ (more than 50 % coverage of the well surface, almost confluent growth with much motility), and 4+ (confluent growth with full motility) (Upcroft and Upcroft 2001). The minimum inhibitory concentration (MIC) was defined as the lowest concentration of each plant nanoemulsion (*Ocimum tenuiflorum*, *Moringa oleifera*, and *Azadirachta indica*) in which a score of 1+ was observed after 48 hours of incubation. Breakpoints

suggested by Upcroft were used (Upcroft and Upcroft 2001). MIC ≤ 1 μg/ml was considered susceptible, MIC = 2 μg/ml was considered intermediate (low-level resistance), and MIC ≥ 4 μg/ml was considered resistant (Upcroft and Upcroft 2001). All experiments were performed in triplicate for each *T. vaginalis* isolate. The same procedure was performed to assess the metronidazole resistance of the isolates.

Culturing of Vaginal Epithelial Cells

Handling Procedures:

Unpacking and storage instructions: All containers were checked for leakage or breakage, and any issues detected were handled with care. The frozen cells were removed from the dry ice packaging and immediately placed at a temperature below -130°C, preferably in liquid nitrogen vapor, until they were ready for use.

Complete Medium Preparation: The base medium for this cell line was ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To create the complete growth medium, fetal bovine serum was added to achieve a final concentration of 10%.

Handling Procedure: To ensure optimal viability, it was recommended to thaw the vial and initiate the culture as soon as it was received. If continued storage was necessary upon arrival, the frozen culture was stored in the liquid nitrogen vapor phase, not at -70°C, to prevent a loss of viability. The vial was thawed by gently agitating it in a 37°C water bath, with care taken to keep the O-ring and cap out of the water. Thawing occurred rapidly, typically within approximately 2 minutes.

Once the contents were thawed, the vial was removed from the water bath, and it was decontaminated by either dipping it in or spraying it with 70% ethanol. All subsequent steps were performed under strict aseptic conditions. The vial contents were transferred to a centrifuge tube containing 9.0 ml of complete culture medium and then centrifuged at approximately 125 x g for 5 to 7 minutes. The cell pellet was resuspended using the recommended complete medium (as per specific batch information

for the culture's recommended dilution ratio) and dispensed into a 75 cm^2 culture flask. Excessive alkalinity of the medium was avoided during recovery, and the culture vessel containing the complete growth medium was placed in the incubator for at least 15 minutes before adding the vial contents to allow the medium to reach its normal pH range (7.0 to 7.6). The culture was then incubated at 37°C in a suitable incubator, with a recommended 5% CO_2 in air atmosphere when using the described medium.

Sub-culturing Procedure

The following volumes were recommended for a 75 cm^2 flask, with adjustments made for vessels of other sizes. Corning® T-75 flasks (catalog #430641) were suggested for sub-culturing this product. The culture medium was removed and discarded, and the cell layer was briefly rinsed with 0.25% (w/v) Trypsin-0.53 mM Ethylenediaminetetraacetic acid (EDTA) solution to remove all traces of serum containing trypsin inhibitor. Next, 2.0 to 3.0 ml of Trypsin-EDTA solution was added to the flask, and the cells were observed under an inverted microscope until the cell layer dispersed, typically within 5 to 15 minutes. Agitation of the cells by hitting or shaking the flask during this process was avoided, and cells that were difficult to detach were incubated at 37°C to facilitate dispersal. Afterward, 6.0 to 8.0 ml of complete growth medium was added, and the cells were aspirated gently by pipetting. Appropriate aliquots of the cell suspension were transferred to new culture vessels, and the cultures were incubated at 37°C. A recommended sub-cultivation ratio ranged from 1:2 to 1:6. Medium renewal was performed 2 to 3 times per week. For cryopreservation, a cryopreservation solution was prepared by supplementing complete growth medium with 100% (v/v) dimethyl sulphoxide (DMSO) (ATCC 4-X).

MTT Assay for Cytotoxicity Assessment

Cell viability was conducted as previously described (Tiloke, Phulukdaree and Chuturgoon, 2013), with slight modifications. The methylthiazol tetrazolium (MTT) assay was used to determine the

cytotoxicity of the plant nanoemulsions on the vaginal epithelial cells. The cells (20, 000 cells/well) were incubated with the nanoemulsions and incubated in a 96-well microtitre plate in triplicate at 37°C, 5% CO_2 for 24 hours. Cells incubated with 100% DMSO was used as a positive control and cells incubated with no media served as the negative control. After 24 hours incubation, CCM/MTT salt solution (5 mg/ml) was added into each well and incubated at 37°C for 4 hours. The supernatant was then removed and 100 µl/well DMSO was added followed by incubation (1 h). The absorbance of the produced formazan was measured at 570 nm and reference wavelength of 690 nm using a Bio Tek µQuant spectrophotometer.

Statistical Analysis

Data Handling: Data obtained from MIC assays and MTT assays were analyzed statistically using appropriate software (e.g., GraphPad Prism). GraphPad Prism V5.0 software was used to plot a concentration-response curve which was subsequently used to determine an IC_{50} value of the plant nanoemulsions on the vaginal epithelial cells.

Results

The MICS of *T. vaginalis* isolates were determined both in the absence and presence of the nanoemulsions at varying concentrations (30 µM, 60 µM, and 120 µM). In the absence of nanoemulsions, *T. vaginalis* isolates 253 and 270 exhibited resistance with an MIC of 4 µg/ml for all three plant extracts. Isolate 101 showed intermediate susceptibility with an MIC of 2 µg/ml for *Ocimum tenuiflorum*, 1 µg/ml for *Moringa oleifera* and *Azadirachta indica*. Isolate 179 demonstrated susceptibility to all plant extracts with an MIC of 1 µg/ml. Notably, in the presence of the nanoemulsions, a loss of resistance was observed. At the 30 µM nanoemulsion concentration, isolates 253 and 270 shifted from resistance to intermediate susceptibility (MIC 2 µg/ml), while isolate 101 remained susceptible (MIC 1 µg/ml). At the 60 µM and 120 µM nanoemulsion concentrations, all isolates displayed susceptibility (MIC 1 µg/ml) (Table 1).

Table 1: Nanoemulsion susceptibility data in the presence and absence of varying concentrations of different plant nanoemulsion.

Plant	***T. vaginalis* isolate**	**MIC in absence of nanoemulsions (µg/ml)**	**Susceptibility Interpretation in absence of nanoemulsions (µg/ml)**	**Nanoemulsion concentration (µM)**	**MIC in presence of nanoemulsions (µg/ml)**	**Susceptibility Interpretation in presence of nanoemulsions (µg/ml)**
Ocimum tenuiflorum	*T. vaginalis* Isolate 253	4	Resistant	30	2	Intermediate
				60	2	Intermediate
				120	1	Susceptible
	T. vaginalis Isolate 270	4	Resistant	30	2	Intermediate
				60	2	Intermediate
				120	1	Susceptible
	T. vaginalis Isolate 101	2	Intermediate	30	1	Susceptible
				60	1	Susceptible
				120	1	Susceptible
	T. vaginalis isolate 179	1	Susceptible	30	1	Susceptible
				60	1	Susceptible
				120	1	Susceptible
Moringa oleifera	*T. vaginalis* Isolate 253	4	Resistant	30	1	Susceptible
				60	1	Susceptible
				120	1	Susceptible
	T. vaginalis Isolate 270	4	Resistant	30	1	Susceptible
				60	1	Susceptible
				120	1	Susceptible
	T. vaginalis	2	Intermediate	30	1	Susceptible
				60	1	Susceptible

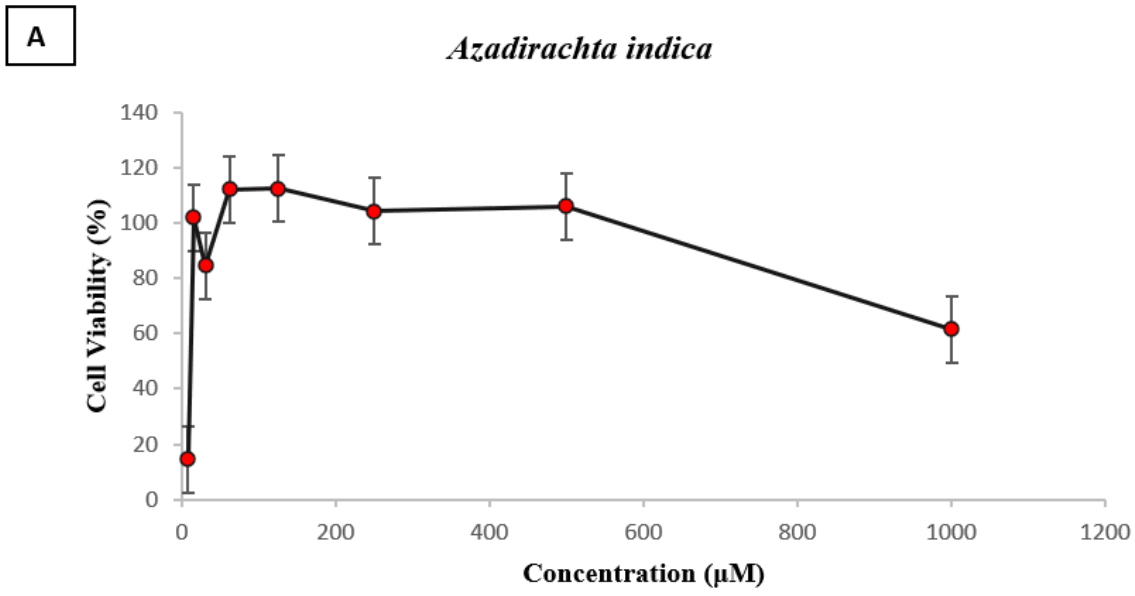
	Isolate 101			120	1	Susceptible
	T. vaginalis isolate 179	1	Susceptible	30	1	Susceptible
				60	1	Susceptible
				120	1	Susceptible
Azadirachta indica	*T. vaginalis* Isolate 253	4	Resistant	30	2	Intermediate
				60	1	Susceptible
				120	1	Susceptible
	T. vaginalis Isolate 270	4	Resistant	30	2	Intermediate
				60	1	Susceptible
				120	1	Susceptible
	T. vaginalis Isolate 101	2	Intermediate	30	1	Susceptible
				60	1	Susceptible
				120	1	Susceptible
	T. vaginalis isolate 179	1	Susceptible	30	1	Susceptible
				60	1	Susceptible
				120	1	Susceptible

At the highest concentration tested (1000 µM), the *Azadirachta indica* extract exhibited a cytotoxic effect, with only 61.60% of cells remaining viable, indicating potential cell damage or death. However, as the concentration of the *Azadirachta indica* extract decreased, there was a notable increase in cell viability, reaching its peak at 125 µM and 62.5 µM, where cell viability exceeded 112%. This suggested that the *Azadirachta indica* extract at these concentrations may have a stimulatory or protective effect on the cells, enhancing their viability. Intriguingly, at 15.625 µM, there was an unexpected increase in cell viability to approximately 101.84%, indicating a potential hormetic response. Conversely, at the lowest concentration tested (7.8125 µM), the *Azadirachta indica* extract exhibited strong cytotoxicity, with only 14.60% of cells remaining viable (Figure 3).

The results demonstrated a concentration-dependent impact on cell viability. At the highest concentration tested (1000 µM), the *Moringa oleifera* extract surprisingly led to an increase in cell viability, with 104.21% of cells remaining viable. This unexpected result suggested a potential stimulatory effect of the *Moringa oleifera* extract on cell health, where it not only maintained cell viability but possibly enhanced cellular metabolic activity. As the concentration of the *Moringa oleifera* extract was reduced, the trend in cell viability varied. At 500 µM, cell viability was significantly higher than 100% (153.83%), indicating an enhanced metabolic state of the cells. This stimulatory effect persisted at concentrations of 250 µM and 125 µM, with cell viability levels at 119.51% and 114.03%, respectively, suggesting a consistent positive influence on cellular health. However, at concentrations below 125 µM, a reversal in the trend was observed. At 62.5 µM and 31.25 µM, cell viability remained relatively high but showed a slight decrease (121.22% and 115.40%, respectively). This could indicate that, beyond a certain concentration threshold, the *Moringa oleifera* extract may have exerted cytotoxic effects on the cells. The most striking change occurred at concentrations of 15.625 µM and 7.8125 µM, where cell viability dropped significantly to 39.49% and 10.60%, respectively, indicating strong cytotoxicity. This sharp decrease in cell viability at the lowest concentrations suggested that the *Moringa oleifera* extract, when present at such low levels, may induce cell damage or metabolic dysfunction (Figure 3).

At the highest concentration tested (1000 µM), the *Ocimum tenuiflorum* extract yielded a negative percentage viability (-3.57%), indicating a detrimental effect on cell health. This concentration of the *Ocimum tenuiflorum* extract seemed to have induced significant cell damage or metabolic dysfunction, leading to a decrease in cell viability. As the concentration of the *Ocimum tenuiflorum* extract was reduced, there was a substantial improvement in cell viability. At 500 µM, cell viability reached 91.01%, suggesting a potential protective or stimulatory effect at this concentration. This result implied that the *Ocimum tenuiflorum* extract, when administered at a moderate concentration, may promote cell health or have antioxidant properties that counteract cellular damage. The dose-dependent

improvement in cell health continued with decreasing concentrations. At 250 µM and 125 µM, cell viability levels were 45.00% and 38.59%, respectively, indicating that lower concentrations of the *Ocimum tenuiflorum* extract still had a positive impact on cell viability. However, an intriguing observation was that, as the concentration decreased further to 62.5 µM and 31.25 µM, cell viability remained relatively stable around 36.30% and 36.73%, respectively. This suggested that the *Ocimum tenuiflorum* extract at these concentrations may have reached a plateau in terms of its beneficial effects on cell health, as there was minimal improvement compared to the higher concentrations. The lowest concentrations of the *Ocimum tenuiflorum* extract (15.625 µM and 7.8125 µM) yielded cell viability levels of 34.09% and 33.62%, respectively. While these concentrations still showed some protective effects compared to the negative control, they did not demonstrate as strong a positive impact as the 500 µM concentration (Figure 3).



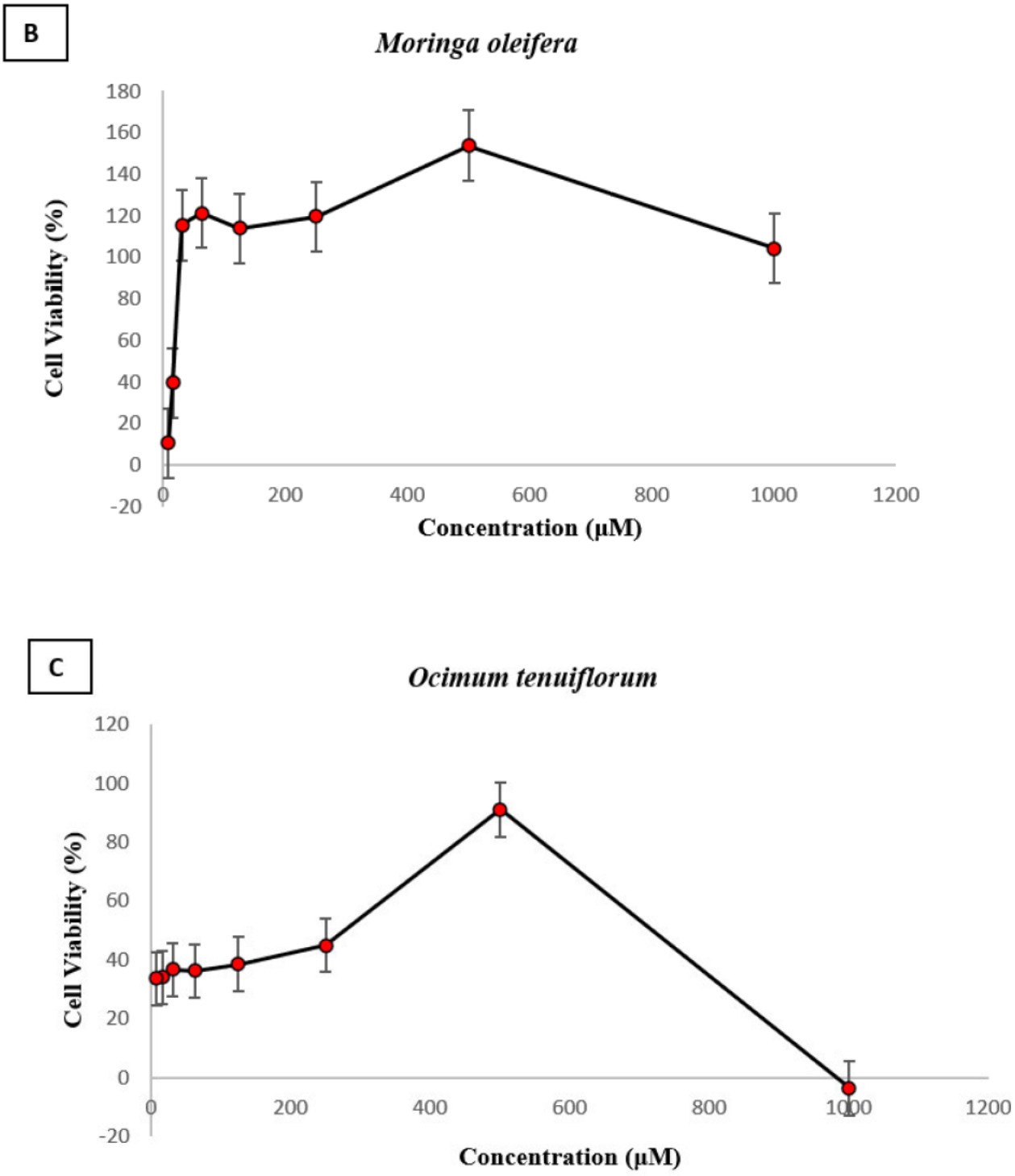


Figure 3: Dose-response curve showing the cytotoxic effect of plant nanoemulsions on Vaginal epithelial cells. (A) The effect of *Azadirachta indica*, (B) *Moringa oleifera* and (C) *Ocimum tenuiflorum* on the cell line.

The IC_{50} values for the nanoemulsions of *Azadirachta indica*, *Moringa oleifera*, and *Ocimum tenuiflorum*on vaginal epithelial cells revealed distinct cytotoxic effects. *Azadirachta indica* exhibited the highest IC50 value at 4318.46 µM, indicating that it necessitates a relatively high concentration to reduce cell viability by 50%. This suggested that *Azadirachta indica* may have a weaker cytotoxic impact on these cells compared to the other substances. In contrast, *Moringa oleifera* showed a significantly lower IC_{50} value of 11 µM, indicating a potent inhibitory effect as only a small concentration is needed for the same 50% reduction in cell viability. *Ocimum tenuiflorum* fell in between with an IC_{50} value of 338.919 µM, signifying a moderate cytotoxic impact on vaginal epithelial cells when compared to both *Moringa oleifera* and *Azadirachta indica* (Table 2).

Table 2: 1. IC_{50} values of the nanoemulsions on the vaginal epithelial cell line after 24 h treatment.

Vaginal epithelial cells	
Azadirachta indica	4318.46 µM
Moringa oleifera	11 µM
Ocimum tenuiflorum	338.919 µM

N = 3 replicates at 95% confidence interval

Discussion

The findings of this study are of paramount significance within the context of South Africa's growing public health challenge posed by trichomoniasis, especially in light of the emergence of metronidazole resistance as a critical issue (Mabaso and Abbai, 2021). Trichomoniasis has become a formidable concern in the country, and this is exacerbated by the coexistence of other prevalent infections such as HIV/AIDS and tuberculosis (de Waaij *et al.,* 2017). The interconnectedness of these diseases has far-reaching implications for both individual and public health. It has been well-documented that individuals infected with trichomoniasis may be at a heightened risk of contracting HIV, and the presence of both infections can lead to more severe health consequences (Masha *et al.,* 2019).

Therefore, the study's findings, particularly in the face of metronidazole resistance, are essential for addressing this intricate healthcare scenario in South Africa. The initial observation that *T. vaginalis* isolates 253 and 270 exhibited resistance to metronidazole in the absence of nanoemulsions, with a MIC of 4 µg/ml, underscores the gravity of metronidazole resistance in the region. This resistance poses a substantial challenge to the traditional treatment methods primarily reliant on metronidazole. This observation aligns with the broader global trend of antimicrobial resistance (AMR) in infectious diseases, a concern that has been extensively discussed in the scientific literature (Graves *et al.,* 2023). AMR not only diminishes the effectiveness of existing treatments but also necessitates a proactive approach in developing novel therapeutic alternatives, as highlighted by the World Health Organization (WHO) and numerous studies (Murugaiyan *et al.,* 2022; WHO, 2020). Furthermore, the varying susceptibility of *T. vaginalis* isolate 101 to different plant extracts in the absence of nanoemulsions, with a MIC of 2 µg/ml for *Ocimum tenuiflorum* and 1 µg/ml for *Moringa oleifera* and *Azadirachta indica*, raises intriguing questions about the adaptability of this parasite and the potential for personalized treatment strategies. This observation aligns with the growing body of literature on the genetic diversity and phenotypic variability of *T. vaginalis* isolates. Studies have shown that different isolates of *T. vaginalis* can exhibit diverse responses to drug treatments due to variations in their genetic makeup (Conrad *et al.,* 2012; Kissinger, 2015). This highlights the importance of tailoring treatment approaches to individual isolates, a concept that is increasingly recognized as crucial in the era of precision medicine (Krzyszczyk *et al.,* 2018).

The introduction of nanoemulsions at varying concentrations (30 µM, 60 µM, and 120 µM) and their significant impact on the susceptibility of *T. vaginalis* isolates represents a remarkable development in the search for alternative treatments, particularly in the context of metronidazole-resistant strains. This phenomenon is particularly compelling in light of antimicrobial resistance (AMR) and the need for innovative solutions to combat resistant pathogens. The observed transition of *T. vaginalis* isolates 253 and 270 from resistant to intermediate susceptibility at a 30 µM nanoemulsion concentration, with

a corresponding decrease in the MIC from 4 µg/ml to 2 µg/ml, suggests a promising synergy between the nanoemulsions and plant extracts. This phenomenon may be attributed to several factors, including the enhanced solubility and bioavailability of bioactive compounds from the plant extracts when incorporated into nanoemulsions (Teixé-Roig, 2023). Research has shown that nanoemulsions can improve the delivery of poorly water-soluble compounds, potentially increasing their therapeutic efficacy (Lawrence and Rees, 2000). Furthermore, the observation that isolate 101 remained susceptible at a 30 µM nanoemulsion concentration, with a MIC of 1 µg/ml, emphasizes the importance of tailoring treatment approaches to individual isolates. The findings here again align with the concept of precision medicine, which has gained traction in recent years as a way to optimize treatment outcomes, particularly in the context of infectious diseases (Watkins, 2022). At higher nanoemulsion concentrations of 60 µM and 120 µM, where all isolates demonstrated susceptibility with a MIC of 1 µg/ml, the potential of nanoemulsions to restore the efficacy of plant-based treatments against metronidazole-resistant *T. vaginalis* strains becomes particularly evident. This result suggests that nanoemulsions have the capacity to overcome resistance mechanisms in these isolates, possibly by facilitating the efficient delivery of bioactive compounds and enhancing their interactions with the parasite.

The observed concentration-dependent effects of *Azadirachta indica* extract on the viability of vaginal epithelial cells in this study provide valuable insights into its potential pharmacological properties and underscores its complex nature. The results reveal a hormetic response, which is characterized by varying cellular responses at different concentration levels, highlighting the nuanced impact of *Azadirachta indica* extract on cell health (Calabrese and Mattson, 2017). At the highest concentration tested (1000 µM), *Azadirachta indica* extract exhibited a cytotoxic effect, with only 61.60% of cells remaining viable. This cytotoxicity aligns with previous studies that have reported the potential for high concentrations of *Azadirachta indica* extracts to exert adverse effects on cellular health (Paul *et al.,* 2011). Such high concentrations might lead to cellular damage or even cell death, indicating the

need for caution when considering *Azadirachta indica* extract at these levels (Paul *et al.,* 2011). Conversely, as the concentration of *Azadirachta indica* extract decreased, a significant increase in cell viability was observed. The most notable enhancement in cell viability occurred at concentrations of 125 µM and 62.5 µM, where cell viability exceeded 112%. This suggests that *Azadirachta indica* extract, at these concentrations, may have a stimulatory or protective effect on the cells, enhancing their viability. This observation is in line with the principle of hormesis, where low doses of a substance can have a stimulating or beneficial effect on biological systems (Mattson, 2008). The unexpected increase in cell viability at 15.625 µM further emphasizes the complexity of *Azadirachta indica* extract's effects. This concentration-response relationship may involve intricate interactions between *Azadirachta indica* extract components and cellular pathways. Hormetic responses like this have been reported for various natural compounds and are indicative of their multifaceted pharmacological actions (Mattson, 2008). However, the results take a dramatic turn at the lowest concentration tested (7.8125 µM), where *Azadirachta indica* extract exhibited strong cytotoxicity, with only 14.60% of cells remaining viable. This highlights that, beyond a certain threshold, *Azadirachta indica* extract can exert detrimental effects on cell viability. Identifying this threshold and understanding the mechanisms behind the decrease in cell viability at lower concentrations are crucial for determining the safe and effective use of *Azadirachta indica* extract in potential therapeutic applications.

The concentration-dependent impact of *Moringa oleifera* extract on cell viability, as observed in this study, reveals a complex and intriguing relationship between the extract and cellular health. At the highest concentration tested (1000 µM), *Moringa oleifera* extract surprisingly exhibited an increase in cell viability, with 104.21% of cells remaining viable. This unexpected result challenges conventional expectations, suggesting a potential stimulatory effect of *Moringa oleifera* extract on cell health. Such findings are not unprecedented in pharmacology, as certain natural compounds have been reported to exert paradoxical effects at extreme concentrations, possibly due to complex interactions with cellular signalling pathways (Calabrese and Baldwin, 2003). The concentration-dependent trend in cell

viability continued to evolve as the *Moringa oleifera* extract concentration decreased. At 500 µM, cell viability exceeded 100%, reaching 153.83%, indicating an enhancement in cellular metabolic activity. This stimulatory effect was consistent, as concentrations decreased to 250 µM (119.51%) and 125 µM (114.03%), suggesting that *Moringa oleifera* extract consistently exerted a positive influence on cellular health. However, the observed trend took an intriguing turn at concentrations below 125 µM. At 62.5 µM and 31.25 µM, cell viability remained relatively high but exhibited a slight decrease (121.22% and 115.40%, respectively). This subtle decline in cell viability at these concentrations raises questions about the potential concentration threshold beyond which *Moringa oleifera* extract might exert cytotoxic effects on the cells (Potestà *et al.,* 2019). The most notable and unexpected change occurred at concentrations of 15.625 µM and 7.8125 µM, where cell viability dropped significantly to 39.49% and 10.60%, respectively, indicating strong cytotoxicity. This sharp decrease in cell viability at the lowest concentrations suggested that *Moringa oleifera* extract, when present at such minimal levels, may induce cell damage or metabolic dysfunction (Nizioł-Łukaszewska *et al.,* 2020). The biphasic response observed in this study highlights the need for a comprehensive understanding of the dose-response relationship of *Moringa oleifera* extract, considering both its potential stimulatory and cytotoxic effects on cellular health.

The observed concentration-dependent impact of *Ocimum tenuiflorum* extract on cell viability presents a multifaceted relationship between the extract and cellular health. At the highest concentration tested (1000 µM), *Ocimum tenuiflorum* extract displayed an unexpected negative percentage viability (-3.57%), indicating a detrimental effect on cell health. This concentration seems to have induced significant cell damage or metabolic dysfunction, leading to a decrease in cell viability. Such a pronounced cytotoxic effect at high concentrations has been reported for various natural compounds and plant extracts, often attributed to the disruption of cellular homeostasis (Matić *et al.,* 2021). As the concentration of *Ocimum tenuiflorum* extract was reduced, a substantial improvement in cell viability was observed. At 500 µM, cell viability reached 91.01%, suggesting a potential protective or

stimulatory effect at this moderate concentration. These results imply that *Ocimum tenuiflorum* extract, when administered within a certain concentration range, may promote cell health or possess antioxidant properties that counteract cellular damage (Hosseinzadeh *et al.,* 2011). The dose-dependent enhancement of cell health persisted with decreasing concentrations. At 250 µM and 125 µM, cell viability levels were 45.00% and 38.59%, respectively, indicating that lower concentrations of *Ocimum tenuiflorum* extract still had a positive impact on cell viability. These concentrations might enhance cell survival or metabolic activity, aligning with previous studies highlighting the antioxidant and cytoprotective potential of *Ocimum tenuiflorum* compounds (Cohen *et al.,* 2014). An intriguing observation arises as the concentration decreases further to 62.5 µM and 31.25 µM, where cell viability remains relatively stable around 36.30% and 36.73%, respectively. This plateau suggests that *Ocimum tenuiflorum* extract at these concentrations may have reached a saturation point in terms of its beneficial effects on cell health, as there was minimal improvement compared to the higher concentrations. The lowest concentrations of *Ocimum tenuiflorum* extract (15.625 µM and 7.8125 µM) yielded cell viability levels of 34.09% and 33.62%, respectively. While these concentrations still demonstrated some protective effects compared to the negative control, they did not exhibit as strong a positive impact as the 500 µM concentration, indicating a non-linear dose-response relationship. These findings underscore the importance of considering concentration thresholds and nonlinear effects when assessing the impact of *Ocimum tenuiflorum* extract on cell viability, providing valuable insights for further investigations into the mechanisms underlying these concentration-dependent responses.

The study's findings reveal varying levels of cytotoxicity among plant nanoemulsions on vaginal epithelial cells (Table 2). *Moringa oleifera's* nanoemulsion demonstrated the highest cytotoxicity, as indicated by its lowest IC50 value, aligning with existing literature emphasizing *Moringa oleifera's* diverse pharmacological properties, including the presence of bioactive compounds like flavonoids, polyphenols, and glucosinolates, which have demonstrated potent anticancer and antiproliferative

effects (Pareek *et al.,* 2023). Conversely, *Ocimum tenuiflorum* nanoemulsion exhibited moderate cytotoxicity, falling between *Moringa oleifera* and *Azadirachta indica*, which corresponds with previous research recognizing the cytotoxic potential of *Ocimum tenuiflorum* due to constituents such as eugenol and flavonoids (Nisar *et al.,* 2021). *Azadirachta indica* nanoemulsion displayed the weakest cytotoxic effect, requiring a higher concentration to achieve similar results. *Azadirachta indica* has been documented for its various biological activities, though its specific cytotoxic effects can vary based on factors such as extraction methods and the specific bioactive compounds present (Alzohairy, 2016).

Conclusion

This research paper has yielded crucial insights into the potential solutions to the escalating public health challenge of trichomoniasis in South Africa, where metronidazole resistance has become a pressing issue. Trichomoniasis is a formidable concern in the country, further compounded by its coexistence with other prevalent infections such as HIV/AIDS and tuberculosis, creating intricate health scenarios with far-reaching implications. This study offers a ray of hope in addressing trichomoniasis and metronidazole resistance in South Africa by harnessing the potential of plant extracts and nanoemulsions. The findings contribute to the broader discussion on AMR and precision medicine while highlighting the need for continued exploration of natural compounds' pharmacological properties for potential therapeutic use. This research represents a critical step towards more accessible, affordable, and effective healthcare options in the region, addressing a pressing public health imperative.

Future research/recommendations

Future research in the field should delve deeper into the underlying mechanisms governing the concentration-dependent effects of the plant extracts on cell viability, shedding light on their pharmacological actions and safety profiles. Identifying the specific bioactive compounds responsible

for these effects and conducting comprehensive dose-response studies will be instrumental in establishing optimal therapeutic ranges and dosing regimens. Furthermore, exploring potential synergies between plant extracts and nanoemulsions for enhanced efficacy against metronidazole-resistant *T. vaginalis* strains warrants investigation. Extending the research to *in vivo* studies and clinical trials, along with cost-effectiveness analyses, is essential to assess safety and efficacy in real-world settings and diverse patient populations. Long-term monitoring of resistance patterns, engagement with local communities, and sustainability considerations are also pivotal aspects of future research in this area. Collaboration with international partners can expand the impact of these studies, contributing to the development of culturally sensitive and accessible treatments for trichomoniasis and addressing the broader challenges of antimicrobial resistance in healthcare.

Limitations

This study has some notable limitations that warrant consideration. Firstly, the scope of this research was limited to *in vitro* investigations, and while these findings provide valuable insights, they may not fully replicate the complex *in vivo* conditions. Secondly, the study primarily used standard laboratory strains and isolates of *T. vaginalis*, which may not represent the full spectrum of genetic diversity and drug resistance profiles seen in clinical settings. Additionally, the assessment of cytotoxicity was conducted on vaginal epithelial cells, but potential interactions with other cell types or tissues were not explored. Furthermore, the concentration-dependent responses observed in the MTT assay, while intriguing, require further mechanistic investigations to elucidate the underlying processes. Finally, this study did not delve into the long-term effects or potential side effects of the tested plant extracts and nanoemulsions. Future research should address these limitations to provide a more comprehensive understanding of the therapeutic potential of these treatments.

Conflict of interest

The author(s) declare no potential conflicts of interests with respect to the research, authorship, and/or publication of this article.

Funding: National Research Foundation, College of Health Sciences funding (University of KwaZulu-Natal)

Ethical Standards

Ethical approval was obtained from the Biomedical Research Ethic Committee (BREC) (BREC/00003557/2021).

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CHAPTER FIVE

This manuscript was submitted to Parasitology Research

(Submission ID: 88a878e9-3f41-46a7-a8bb-a6c17c41c591)

Nanocomplex-Based siRNA Delivery: Expanding Frontiers in *Trichomonas vaginalis* Infection Control and Precision Medicine

Rowen Govender[a,*], Swenkile Ndlovu[b] and Nathlee S Abbai[a]

[a]Department of Clinical Medicine, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa

[b]Department of Medical Microbiology, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa

[*]***Corresponding author:***

Rowen Govender

Department of Clinical Medicine,

College of Health Sciences,

Nelson R. Mandela School of Medicine,

University of KwaZulu-Natal,

Durban, South Africa

Email: rowengovende@gmail.com; ABBAIN@ukzn.ac.za

ORCID Number: 0000-0003-4820-4574

Abstract

Introduction: The escalating problem of antibiotic resistance and the limitations of current treatments for *Trichomonas vaginalis* infections demand innovative therapeutic approaches. This research delves into the potential of iron nanoparticles (FeNPs) modified with chitosan (Cs) and small interfering RNA (siRNA) for addressing *T. vaginalis* infections while advancing nanomedicine in precision medicine applications.

Methods: Iron nanoparticles were synthesized and characterized via UV spectroscopy to affirm their identity and chitosan conjugation. Transmission Electron Microscopy (TEM) provided insight into the morphological alterations of FeNPs under various conditions. A band shift assay revealed efficient siRNA binding to nanocomplexes. Furthermore, the protective attributes of nanocomplexes were assessed using RNase A-mediated digestion studies. Cell viability studies examined the concentration-dependent effects of these nanocomplexes, and gel electrophoresis was employed to investigate siRNA interactions with *T. vaginalis*.

Results and Discussion: The successful synthesis of FeNPs and chitosan conjugation confirmed the feasibility of these nanocomplexes. Efficient siRNA binding by nanocomplexes holds promise for gene silencing applications. Cell viability studies highlighted both cytotoxic and stimulatory effects, underscoring potential therapeutic applications. Gel electrophoresis revealed promising siRNA interactions with *T. vaginalis*, suggesting innovative approaches for *T. vaginalis* treatment. These findings expand our understanding of drug delivery, gene silencing, and targeted therapies. However, to effectively translate these results, further exploration, *in vivo* validation, and clinical studies are needed.

Conclusion: This study lays a robust foundation for precision medicine. The findings offer prospects in drug delivery, gene silencing, and targeted therapies, but their application will benefit from continued research and clinical evaluation to address the growing challenges of *T. vaginalis* infections.

Keywords: *Trichomonas vaginalis, nanocomplexes, small interfering RNA (siRNA), iron nanoparticles, precision medicine*

Introduction

Trichomonas vaginalis, a flagellated protozoan parasite, serves as the causative agent of trichomoniasis, a globally prevalent sexually transmitted infection (STI) that carries substantial public health implications (1). Trichomoniasis is more than just a common STI; it represents a significant and widespread concern, impacting millions of individuals annually, with severe repercussions for both women and men (2). These consequences include an increased risk of preterm birth, low birth weight in infants, and heightened susceptibility to HIV infection (1).

Metronidazole, a nitroimidazole antimicrobial drug, has long been the primary treatment for trichomoniasis, playing a crucial role in mitigating the spread of the infection (3). However, the worrisome emergence of metronidazole-resistant strains of *T. vaginalis* has cast a shadow of uncertainty over the efficacy of this traditional therapy (4). This alarming development necessitates the exploration of innovative therapeutic strategies capable of countering the adaptability and resilience of this formidable pathogen.

Our current research endeavours to address this critical challenge by proposing a ground-breaking approach that combines the unique attributes of iron nanoparticles (FeNPs) synthesized from the leaves of *Ocimum tenuiflorum* (commonly known as Holy Basil) with the precision of small interfering RNA (siRNA) technology. This amalgamation of FeNPs and siRNA holds the potential to revolutionize our strategy for combating metronidazole-resistant *T. vaginalis* infections.

The adoption of nanoparticles, specifically FeNPs, signifies a paradigm shift from traditional drug delivery methods. Nanoparticles offer a set of intrinsic advantages that make them exceptional vehicles for delivering therapeutic agents (5). Their incredibly small size, typically measured in nanometres, facilitates their seamless entry through biological barriers and into cellular interiors (6). This property allows for precise targeting of therapeutic payloads directly to the site of infection, reducing off-target effects and optimizing therapeutic potency (7).

Ocimum tenuiflorum, esteemed for its medicinal properties across diverse cultures, provides a natural source for synthesizing FeNPs (8). The choice of using *Ocimum tenuiflorum* imparts several advantages. The green synthesis of FeNPs using plant extracts not only capitalizes on the therapeutic potential of phytochemicals but also aligns with sustainable and eco-friendly principles (9). These FeNPs, endowed with unique physicochemical properties, provide an ideal platform for the controlled and sustained release of therapeutic agents. Additionally, *Ocimum tenuiflorum* extracts may contribute supplementary bioactive compounds that enhance the antimicrobial and anti-inflammatory attributes of our therapeutic approach (10).

In addition to the advantages of FeNPs, our strategy incorporates the precision of siRNA technology. Small interfering RNAs enable the targeted silencing of specific genes, allowing us to disrupt crucial molecular pathways essential for *T. vaginalis* pathogenesis (11). This precise gene-silencing approach holds immense promise not only in curtailing the virulence of the parasite but also in mitigating the risk of resistance development.

In this manuscript, we present the conceptual framework and preliminary findings of our ongoing research, aimed at synthesizing *Ocimum tenuiflorum*-mediated FeNPs and conjugating them with custom-designed siRNAs. Together, these components create a robust and multifaceted therapeutic platform that addresses both drug resistance and pathogenicity concurrently. By harnessing the advantages of nanoparticles and siRNA technology, our innovative approach offers new avenues for the effective treatment of metronidazole-resistant *T. vaginalis* infections, thereby significantly improving patient outcomes and advancing public health initiatives. This multifaceted approach holds great potential for addressing the challenges posed by metronidazole-resistant *T. vaginalis* strains while capitalizing on the remarkable attributes of nanoparticles and the medicinal potential of *Ocimum tenuiflorum*.

Methods

Culturing of Trichomonas vaginalis

Two metronidazole-resistant *T. vaginalis* isolates were used in this study. The isolates were cultured from self-collected vaginal swabs obtained from antenatal women receiving care at the King Edward VIII in Durban, South Africa. To maintain culture purity, the stored isolates were grown in tubes containing diamonds TYM media supplemented with antibiotics (ampicillin, amphotericin B, ciprofloxacin, chloramphenicol, vancomycin, and amikacin). The incubation took place over three to five days at 37°C with a controlled atmosphere of 5% CO_2. Routine checks were performed for optimal cell growth via wet mount microscopy. Regular sub-culturing was conducted to ensure culture viability, with isolates considered optimally grown upon the observation of cells exhibiting jerk motility.

Sample Collection and Preparation

Sample Collection

Ocimum tenuiflorum leaves were collected in May 2021 from the Botanical Gardens in Durban (29.8483° S, 31.0083° E), KwaZulu-Natal, South Africa. Young leaves were obtained from the tree and placed in a sealed plastic bag to prevent transpiration and drying during transport to the laboratory.

Preparation of Ocimum tenuiflorum Leaf Extract

The *Ocimum tenuiflorum* plant leaves were prepared following a previously established method [12]. The leaves were carefully washed with distilled water immediately after collection and gently dried with a paper towel. Approximately 10 g of the leaves were then chopped into small pieces and placed in a beaker containing 40 mL of 18 Mohm water. The mixture was heated to a temperature between 80 and 90°C with continuous stirring for 15 minutes, resulting in the formation of a green-coloured

liquid solution. The leaf extract was subsequently filtered through Whatman Filter paper no. 5 and stored at 4°C until further use.

Synthesis of Iron Nanoparticles

To synthesize FeNPs, 0.1 mol/L $FeSO_4$ was added to the plant extract in a 5:1 volume ratio, with stirring continuing until the solution turned dark brown, indicating the reduction of Fe^{2+}. The size of the synthesized FeNPs from *Ocimum tenuiflorum* leaves extracts was assessed by monitoring the FeNPs' visible absorption band using the UV-vis spectrophotometric method.

Functionalization of FeNPs with Chitosan:

A chitosan (CS) solution (1 g/ml in 1% acetic acid) was added to the nanoparticles, with stirring in predetermined ratios of 1:1, 1:2.5, and 1:3.5 v/v for optimal binding.

Preparation of the Chitosan-FeNP:siRNA Nanocomplexes:

Before each assay, all functionalized FeNP preparations were vortexed for approximately 1 minute and sonicated for 15 minutes. Varying amounts of the respective functionalized FeNP suspensions were added to the siRNA (*T. vaginalis* SIRNA2: 5'-gttccagatc tcaagaacca g-3) targeting the *AP-65* gene to obtain a range of mass (w/w) ratios. These nanocomplexes were made up to a final volume of 10 µl in HEPES buffered saline (HBS) and allowed to incubate at room temperature for 1 hour to facilitate electrostatic nanocomplex formation.

Characterization of Bioreduced Iron Nanoparticles

UV-Vis Spectroscopy

UV-Vis spectroscopic analysis was carried out with a cuvette of a path length of 10 mm, and measurements were performed as a function of reaction time at room temperature.

Transmission Electron Microscopy (TEM)

TEM was used to determine the ultrastructural morphology of the Iron Nanoparticles and its functionalized counterparts. Images were captured using a JEOL-JEM T1010 electron microscope and analyzed with iTEM Soft Imaging Systems (SIS) Mega view III (JEOL, Tokyo, Japan). A drop of the respective sample was placed onto a carbon-coated copper grid and air-dried at room temperature, with subsequent viewing, capturing, and analysis of the images.

Nanoparticle Tracking Analysis (NTA)

The NPs were characterized using NTA for a more accurate measurement of their size (nm) and eta (ζ) potential at 24 V at 25°C. A 1:100 dilution of the NPs in ultrapure water was used and assessed using a Nanosight NS500 and NTA 3.0 software (Malvern Instruments, Worcestershire, UK). Prior to sample analysis, the instrument was primed and flushed, with the camera position set at zero. The particle size distribution and zeta potentials were determined using the Stokes-Einstein equation and Smoluchowski approximations, respectively.

Nucleic Acid Binding Studies

Band Shift Assay

The band shift assay was employed to assess the ability of the functionalized nanocomplexes to bind siRNA. Complete complexation was indicated by a net neutral charge, achieved through optimal binding of the negatively charged siRNA and the positively charged nanocomplexes. This optimal ratio inhibited migration through the gel matrix. The nanocomplexes were subjected to an incubation period of 1 hour at 25°C to ensure effective binding of the NP to the siRNA, followed by electrophoresis at 50 V for 90 minutes on a 1% agarose gel.

RNase A Protection Assay

The RNase A-mediated digestion study was used to determine the ability of the functionalized nanoparticles to protect the bound siRNA from enzymatic degradation, employing agarose gel electrophoresis. Nanocomplexes were prepared using 0.3 µg siRNA at sub-optimum, optimum, and supra-optimum mass ratios (w/w), as obtained from the band shift assay. Following a 1-hour incubation period, *RNase A* was added to each reaction mixture to a final concentration of 10% (v/v). The reaction mixtures were subsequently incubated at 37°C for 2 hours in a digital temperature-controlled water bath. After incubation, Ethylenediaminetetraacetic acid (EDTA) (10 mM) and sodium dodecyl sulphate (SDS) (0.5%) were introduced to the reaction mixtures, followed by further incubation for 20 minutes at 55°C, and electrophoresis under the same conditions as previously described.

In vitro Cell-Based Assays

3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) Cell Viability Assay (Iron nanoparticles cytotoxicity against vaginal epithelial cells):

Vaginal epithelial cells at or near confluency were trypsinized (using trypsin/versene) and seeded into 48-well plates at a density of 1.8-2.5 x 10^5 cells/well. Cells were incubated at 37°C for 24 hours in 5% CO_2. Nanocomplexes at varying concentrations (NPs/siRNA) were added to their respective wells in triplicate and incubated at 37°C for 48 hours in 5% CO_2. A positive control containing untreated cells was included. Thereafter, the medium in each well was replaced with 200 µL fresh growth medium (EMEM) containing 20 µL MTT reagent (12 mM in PBS), and cells were incubated at 37°C for 4 hours. The growth medium infused with MTT was then removed, followed by the solubilization of the formazan crystals using 200 µL Dimethyl sulfoxide **(**DMSO). Absorbance at 570 nm was measured using a microplate reader (Mindray MR-96A, Vacutec, Hamburg, Germany).

Cell viability was calculated as follows:

% Cell Viability = $[A570nm\ treated\ Cells]/[A570nm\ untreatedCells]$ x 100

Transfection Analysis

To ensure successful transfection, the stepwise Lipofectamine RNAiMAX Reagent protocol was followed over two days. On the first day, *T. vaginalis* cells were seeded in 96-well plates to achieve 60-80% confluence by the time of transfection, using the specified cell quantities for each plate size as per the manufacturer's instructions. On subsequent days, the transfection steps were carried out. First, Nanoparticle+siRNA-lipid complexes were prepared by diluting Lipofectamine® RNAiMAX Reagent and nanoparticle+siRNA in Opti-MEM according to the recommended volumes and ratios. The Nanoparticle+siRNA-lipid complex was incubated for 5 minutes at room temperature. Following this, the Nanoparticle+siRNA-lipid complex was added to the plated cells in volumes and quantities corresponding to the plate size. After the addition, the transfected cells were incubated for 3 days at 37°C. This protocol was thoughtfully designed to accommodate triplicate transfections, all while accounting for potential pipetting variations. Lastly, an analysis was conducted to assess the transfection efficiency.

Agarose gel electrophoresis of siRNA before and after treatment with *T. vaginalis* cells was conducted to assess nanoparticle+siRNA attachment to the cells. This method involved the separation of siRNA molecules based on their size and charge using novel juice for band visualization. In the experiment, a 1% agarose gel was prepared, and siRNA samples (before and after treatment with *T. vaginalis* cells) were loaded. Electrophoresis was conducted at 90V for 30 minutes. The siRNA bands were visualized using a gel documentation system. The analysis involved the examination of siRNA bands in all lanes. Successful siRNA attachment to *T. vaginalis* cells was indicated by a decrease in the intensity or migration of the siRNA band in the "after treatment" lane compared to the "before treatment" lane, providing visual confirmation of siRNA attachment and validating the effectiveness of the treatment.

MTT Cell Viability Assay for Treated and Untreated Trichomonas vaginalis

The MTT assay was conducted as follows: Initially, vaginal epithelial cells were maintained in a suitable cell culture medium, which contained 10% FBS and 1% penicillin-streptomycin. These cells were cultured in 75 cm^2 flasks and kept at 37°C with 5% CO_2, ensuring that they were in the exponential growth phase.

For the assay, an appropriate number of cells were used, with typically 5,000 cells seeded into each well of 96-well plates in 100 µL of culture medium. These cells were detached using 0.25% trypsin-EDTA and neutralized with culture medium. Prior to seeding, the cells were washed gently with PBS, and the culture medium was aspirated.

Control groups without treatment were included to provide a baseline for comparison. Iron nanocomplexes with siRNA were prepared at different concentrations for the treatment groups. These concentrations were obtained by diluting a stock solution of the nanocomplexes with culture medium, ranging from 1000 uM to 7.8125 uM. After cell seeding, the prepared iron nanocomplexes were added to the respective wells, and the plates were incubated at 37°C with 5% CO_2 for a defined period, commonly, 24 hours. Following incubation, the culture medium was aspirated from each well, and 100 µL of MTT reagent, typically at a concentration of 0.5 mg/mL, was added to initiate the formation of purple formazan crystals over a 3-4-hour incubation period. Subsequently, the MTT solution was carefully aspirated from each well, and 100 µL of DMSO was added to dissolve the formazan crystals. The plate was gently shaken to ensure complete dissolution. Finally, the absorbance at 570 nm was measured using a microplate reader, and the results were analysed to assess the impact of iron nanocomplexes with siRNA on vaginal epithelial cell viability.

Results

UV spectroscopy was harnessed to elucidate the identity of iron nanoparticles and to validate the successful conjugation of chitosan (Cs) to these nanomaterials. The graphical representation of our findings displayed absorbance against wavelength and revealed two distinct peaks of significance (Figure 1). The first, at a wavelength of 273 nm, was instrumental in the identification of iron nanoparticles, reflecting their characteristic light-absorbing properties. This peak served as a definitive marker of the presence and concentration of these nanoparticles within the sample. The second peak, observed at 269 nm, indicated the outcome of chitosan conjugation. This shift in the peak from 273 nm (representing unconjugated iron nanoparticles) to 269 nm (signifying conjugation) provided compelling evidence of the effective binding of chitosan to the iron nanoparticles, underlining the success of this crucial modification. These UV spectroscopy results not only confirmed the presence of iron nanoparticles but also validated the chitosan conjugation.

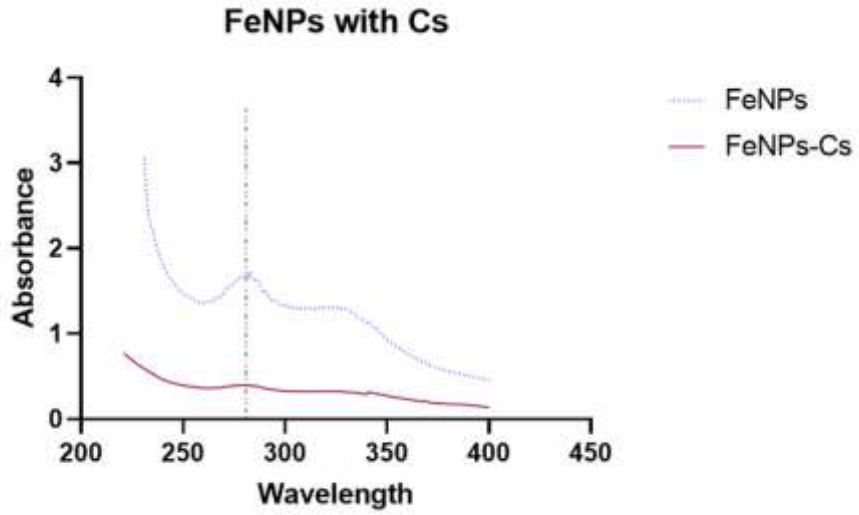

Figure 1: UV Spectroscopy Analysis of Iron Nanoparticles and Chitosan Conjugation

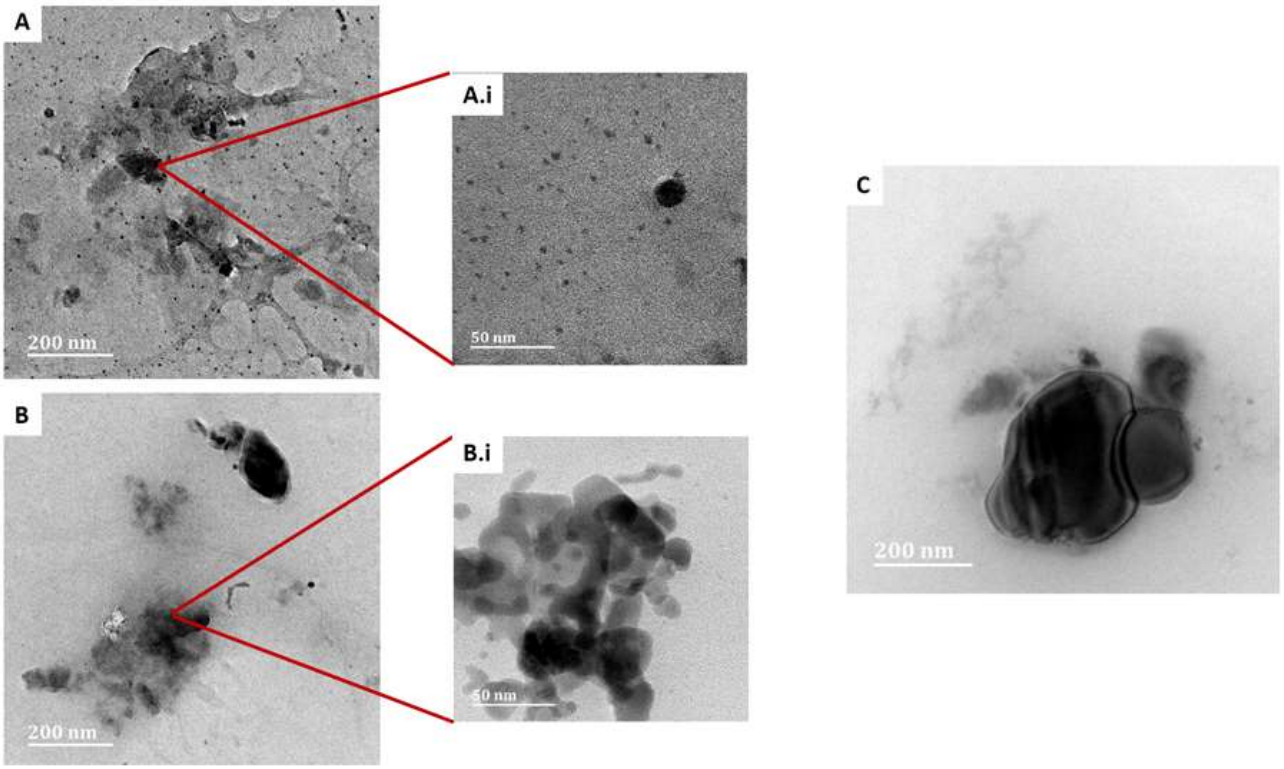

Figure 2: Transmission Electron Microscopy (TEM) Analysis of Iron Nanoparticles (FeNPs)

In Figure 2, we present TEM images providing an in-depth examination of the structural morphology, size, and dispersity of the iron nanoparticles (FeNPs). These images reveal crucial insights into the characteristics of the nanoparticles. In Figure 2A and its zoomed-in version (2Ai), the FeNPs appear as smooth, spherical particles, falling within the size range of 40 nm to 60 nm. Their average size is measured at 50.6 ± 9.4 nm (as detailed in Table 1). Moving to Figure 2B and its zoomed counterpart (2Bi), the introduction of chitosan results in the formation of clustered FeNPs while maintaining their initial spherical shape, albeit with an increased size. These clustered nanoparticles have an average size of 95.2 ± 17.5 nm, spanning a range from 77.7 nm to 110 nm. Finally, in Figure 2C, we observe the successful conjugation of FeNPs with chitosan and siRNA, resulting in a composite structure with an average size of 117.3 ± 11.9 nm, ranging from 105.4 nm to 129.2 nm.

Table 1: Particle size (nm) obtained from TEM imaging (*n*=3)

Sample	**Particle Size (nm) ± SE**
FeNP	50.6 ± 9.4
FeNP + Cs	95.2 ± 17.5
FeNP + Cs + siRNA	117.3 ± 11.9

The results in Table 1 illustrate the particle sizes, in nanometres (nm), determined through TEM imaging for three distinct samples. In the first sample, FeNP, the average particle size was found to be 50.6 nm, with a standard error (SE) of 9.4 nm, indicative of a relatively precise measurement. When chitosan was introduced alongside FeNP in the second sample (FeNP + chitosan), the particle size increased to 95.2 nm, with a larger SE of 17.5 nm, signifying greater variability in particle size within the sample. In the third sample (FeNP + chitosan + siRNA), which included both FeNP and chitosan as well as siRNA, the average particle size further increased to 117.3 nm, with a slightly smaller SE of 11.9 nm compared to the second sample. These results suggest that the addition of chitosan and siRNA to the FeNP sample influences particle size, and the SE values offer insights into the precision and reproducibility of the measurements.

Table 2: Particle size (nm) and zeta potential (mV) of the nanoparticles from Nanoparticle Tracking Analysis.

	Particle Size (nm) ± SE		ζ-potential (mV) ± SE	
Sample	Nanoparticle	Nanocomplex (NP + siRNA)	Nanoparticle	Nanocomplex (NP + siRNA)
FeNP	64.8 ± 14.5	-	-3.7 mV ± 0.2	-
FeNP + Cs	113 ± 4.3	186.8 ± 16.9	22.1 ± 0.5	12.4 mV ± 0.5

Table 2 summarizes the results of Particle Size (in nanometres[nm]) and Zeta Potential (in millivolts [mV]) obtained through Nanoparticle Tracking Analysis for a set of nanoparticles in their native state (Nanoparticle) and when complexed with small interfering RNA (siRNA) to form nanocomplexes (NP

+ siRNA). For FeNP (Sample 1), the average particle size is 64.8 nm with a standard error of 14.5 nm, and the zeta potential is -3.7 mV with a standard error of 0.2 mV. In contrast, FeNP + Cs (Sample 2), which includes chitosan (Cs), shows significant alterations. The particle size increases to 186.8 nm with a standard error of 16.9 nm when complexed with siRNA, and the zeta potential decreases to 12.4 mV with a standard error of 0.5 mV. These findings suggest that the addition of chitosan and siRNA to the FeNP samples has a substantial impact, resulting in larger particle sizes and changes in surface charge. These data are vital for understanding how these nanoparticles may function and interact in various applications, particularly those involving siRNA delivery and targeted therapies.

DNA Binding studies

Electrophoretic Mobility shift assay

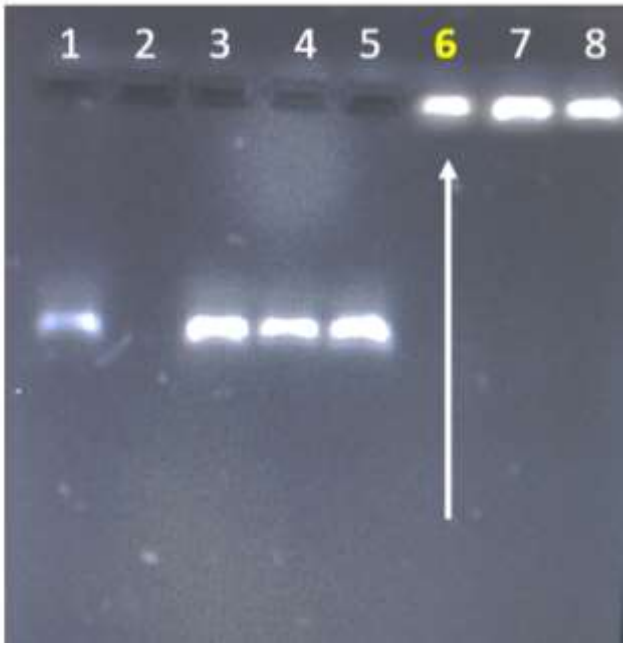

Figure 3: Band Shift Assay Results for Nanocomplexes Binding with siRNA

In the band shift assay conducted to assess the binding capability of functionalized nanocomplexes with siRNA, the gel electrophoresis results in each lane offered valuable insights. Lane 1, the positive control containing only siRNA (0 microliters of nanoparticles), exhibited siRNA migration,

demonstrating the mobility of unbound siRNA. Conversely, Lane 2, the negative control with only nanoparticles (0 microliters of siRNA), showed no siRNA bands, confirming that nanoparticles alone did not generate bands on the agarose gel. Lanes 3 to 5, with nanoparticle volumes of 0.001, 0.01, and 0.1 microliters respectively, displayed migrating siRNA bands, indicating incomplete binding. However, in Lanes 6 to 8, with nanoparticle volumes of 0.002, 0.02, and 0.2 microliters respectively, siRNA bands did not migrate. Notably, Lane 6 showed optimum binding, demonstrating that at this specific nanoparticle-to-siRNA ratio, effective binding occurred, preventing siRNA from migrating through the gel. These findings elucidate the conditions under which nanocomplexes can efficiently bind siRNA, a vital aspect in siRNA delivery and targeted therapeutic applications.

Nuclease Protection Assay

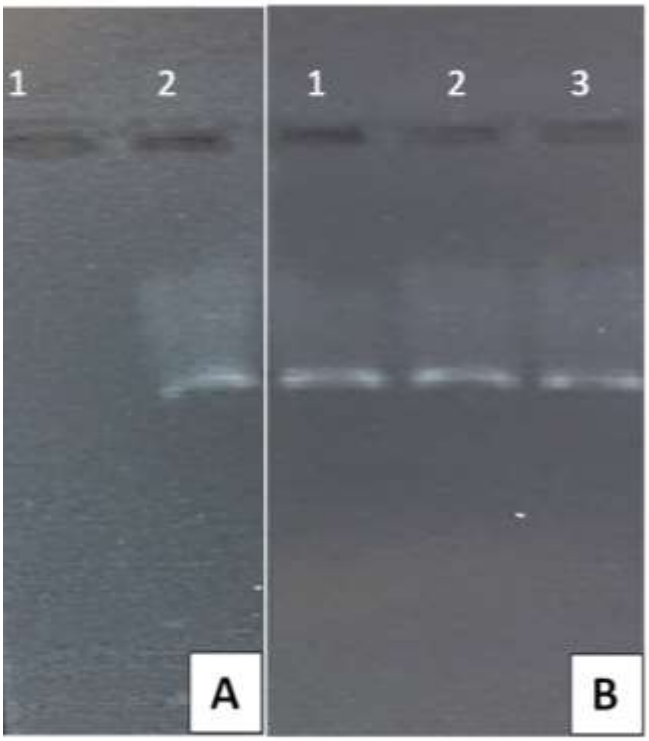

Figure 4: RNase A-Mediated Digestion Study of Nanocomplexes

In our RNase A-mediated digestion study, nanocomplexes were formed using siRNA at various mass ratios, from sub-optimum to supra-optimum as determined by the band shift assay. When subjected to

RNase A, the negative control (Lane 1) exhibited complete degradation of siRNA, evident by the absence of any visible bands on the gel. The positive control (Lane 2), where siRNA was treated with nanoparticles but not with *RNase A*, showed a distinct migrated band, indicating that the nanoparticles alone did not degrade the siRNA (Figure 4A). Surprisingly, in Lanes 1 to 3, where nanoparticles were introduced at volumes ranging from 0.001 to 0.003 microliters, migrated bands were still visible. Contrary to the expectation, these bands indicated that the nanoparticles did protect the siRNA from degradation by *RNase A*, allowing the siRNA to remain intact and visible on the gel. In the positive control, the presence of a band highlighted the protective effect of nanoparticles on siRNA stability, preventing its degradation even in the presence of *RNase A* (Figure 4B). These findings underscore the potential of these nanoparticles in safeguarding siRNA integrity, crucial for the success of gene silencing and targeted therapeutic applications under enzymatic challenges.

MTT Cell Viability assay

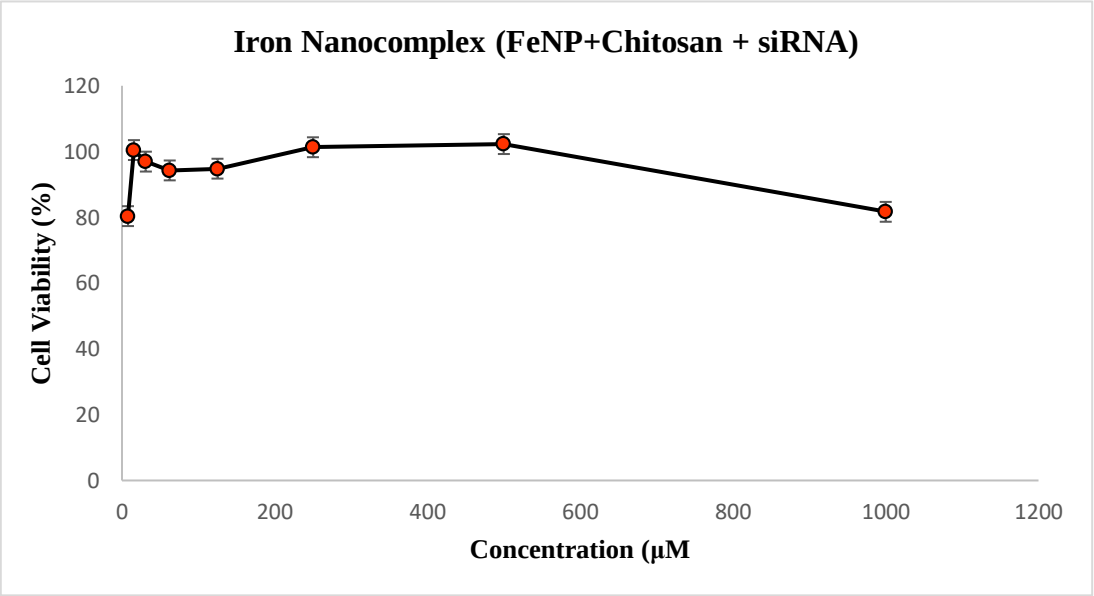

Figure 5: Impact of Iron Nanoparticles, Chitosan, and siRNA on Vaginal Epithelial Cell Viability

This study evaluated the impact of iron nanoparticles combined with chitosan and siRNA on the viability of vaginal epithelial cells, presenting the results in terms of concentration (uM) and

percentage viability. At a high concentration of 1000 uM, the nanoparticles exhibited some toxicity, with cell viability around 81.67%. However, as the nanoparticle concentration decreased, a shift in effects was observed. At 500 uM and 250 uM, cell viability improved, reaching 102.27% and 101.31%, suggesting potential stimulatory effects. Subsequent concentrations of 125 uM, 62.5 uM, and 31.25 uM demonstrated stable cell viability of approximately 94.79%, 94.26%, and 96.94%, respectively. At lower concentrations of 15.625 uM, cell viability increased to 100.45%, possibly indicating a stimulatory response, while at 7.8125 uM, a minor decrease to 80.37% was observed. These findings highlight a concentration-dependent influence of the nanoparticles on vaginal epithelial cell viability, providing valuable insights into their biocompatibility and potential effects. These results contribute to our understanding of the safety and suitability of these nanoparticles for applications such as drug delivery and therapeutic interventions in the context of vaginal health.

Transfection Analysis

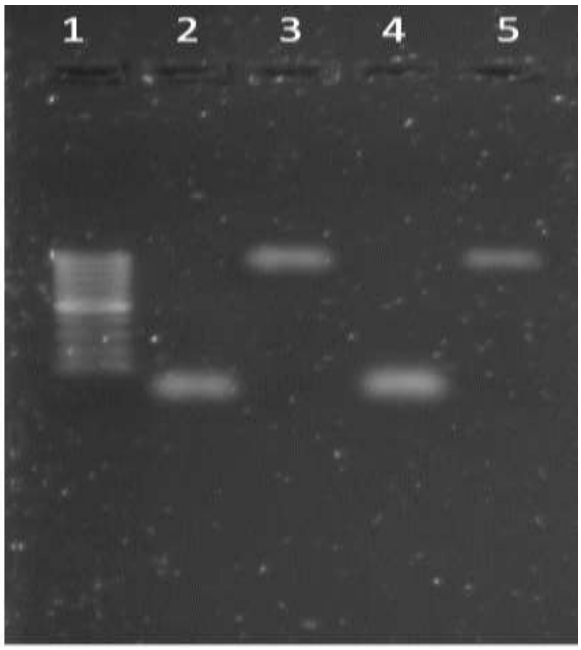

Figure 6: Gel Electrophoresis Analysis of siRNA, Nanocomplexes, and *T. vaginalis*

In Figure 6, a 100bp DNA ladder was used as a reference marker in Lane 1, verifying the proper functionality of the electrophoresis process. Lane 2, which contained siRNA alone, demonstrated siRNA migration beyond the 100bp DNA ladder, consistent with its anticipated small size. Notably for Lanes 3 and 5, siRNA which was combined with nanocomplexes and samples of *T. vaginalis* (sample 253 and sample 270, respectively), exhibited a distinct migration pattern. In both instances, the siRNA mobility was reduced to approximately half that of siRNA alone (Lanes 2 and 4). This alteration in migration behaviour strongly suggests a successful interaction between the siRNA, nanocomplexes, and *T. vaginalis* samples, indicating complex formation or binding to the *T. vaginalis* DNA.

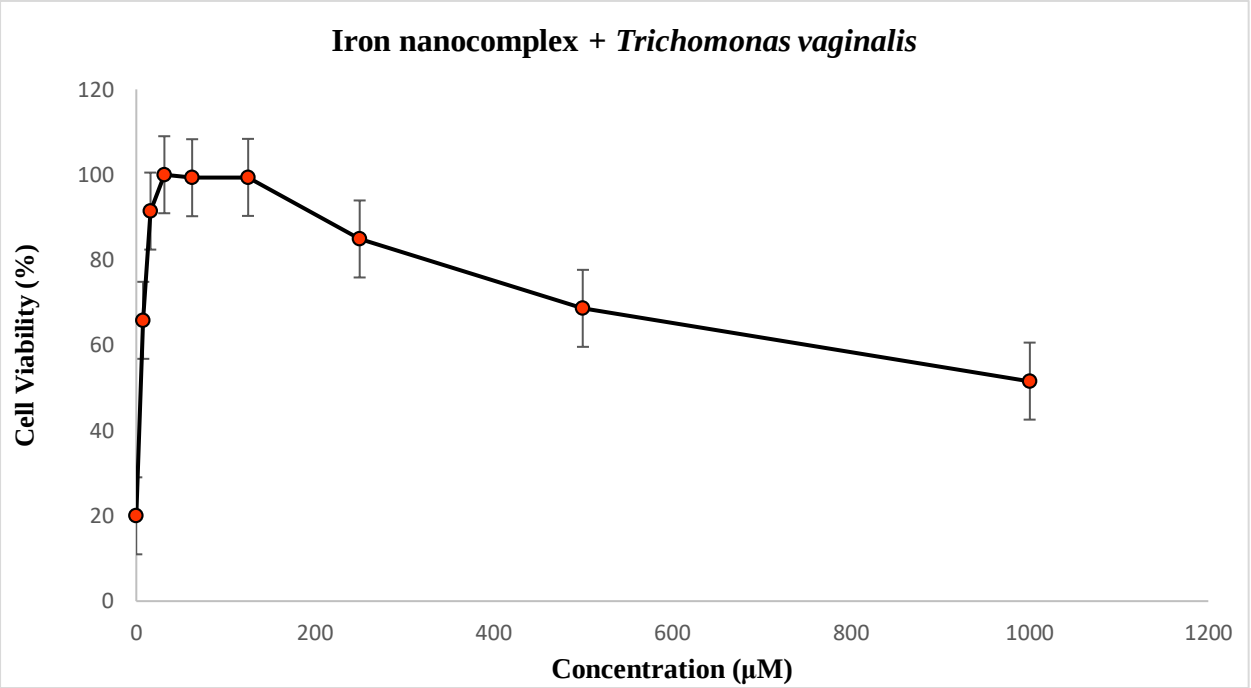

Figure 7: Impact of Iron Nanocomplexes and *T. vaginalis* on Vaginal Epithelial Cell Viability

The results indicate a significant shift in cell viability as the concentration of iron nanocomplexes with *T. vaginalis* decreases. At the highest concentration of 1000 µM, cell viability is notably reduced to approximately 51.58%. This outcome suggests that at high concentrations, the combination of iron

nanocomplexes with *T. vaginalis* exerts a considerable cytotoxic effect, which could potentially be attributed to the presence of *T. vaginalis* and its adherence to the cells.

As the concentration decreases to 500 µM and 250 µM, cell viability progressively improves to around 68.64% and 84.92%, respectively, indicating a reduction in cytotoxicity. However, the most intriguing observation is seen at 125 µM. At this concentration, cell viability remarkably increases to almost 99.35%. This marked recovery in cell viability suggests the successful silencing of the adhesion gene by the siRNA, preventing *T. vaginalis* from adhering to the vaginal epithelial cells and thereby protecting them from cytotoxicity.

Subsequent concentrations, ranging from 62.5 µM to 15.625 µM, continue to demonstrate high cell viability, with values approaching or exceeding 99%. This consistency suggests that the combination of iron nanocomplexes with *T. vaginalis*, at these concentrations, remains minimal. At the concentration of 7.8125 µM, there is indeed a noticeable decrease in cell viability to approximately 65.84%. This is an important finding and indicates that at this specific concentration, the combination of iron nanocomplexes with *T. vaginalis* appears to have a cytotoxic effect on the vaginal epithelial cells. While the previous concentrations demonstrated minimal impact on cell viability, the outcome at 7.8125 µM suggests that the cytotoxicity becomes more pronounced at this lower concentration.

Notably, the results at 0 µM (control condition, representing *T. vaginalis* treatment alone) indicate a baseline cell viability of 20%, which aligns with expectations. In this control group, where the adhesion gene silencing is not at play, *T. vaginalis* effectively infects the vaginal epithelial cells and causes substantial cell death.

Discussion

The results presented in Figure 1 from the UV spectroscopy analysis played a pivotal role in affirming the identity of iron nanoparticles and confirming the successful conjugation of chitosan, both of which hold significant implications for various biomedical applications. The UV absorbance spectra depicted

two distinct peaks which were highly significant. The first peak, observed at a wavelength of 273 nm, aligns with prior studies (13) on iron nanoparticles, substantiating their characteristic light-absorbing properties. This peak serves as a definitive marker for the presence and concentration of iron nanoparticles within the sample, a crucial verification step for the successful synthesis of these nanomaterials. Moreover, the results corroborate existing literature, thus strengthening our confidence in the characterization of these nanoparticles. The second peak, at 269 nm, offers strong evidence of chitosan conjugation to the iron nanoparticles. This shift in the peak from 273 nm (representing unconjugated iron nanoparticles) to 269 nm (indicating conjugation) aligns with the findings of earlier studies (14), demonstrating the binding of chitosan to the iron nanoparticles. This UV spectroscopy analysis not only serves to confirm the presence of iron nanoparticles but also validates the effective conjugation of chitosan, a milestone achievement in the development of functionalized nanomaterials with broad implications. This successful conjugation is well-documented in the literature and finds applications in diverse fields, including drug delivery systems (15), which leverage the unique properties of chitosan to facilitate targeted therapeutic interventions.

The utilization of iron nanoparticles (FeNPs) as antimicrobial agents holds great promise, supported by a growing body of literature. The high surface area and broad-spectrum antimicrobial activity of FeNPs make them formidable contenders in the fight against microbial infections (16). Their ability to generate reactive oxygen species (ROS), disrupt biofilms, and mitigate antimicrobial resistance aligns with the critical need for effective and versatile antimicrobial strategies (17). Additionally, FeNPs can be engineered for biocompatibility and sustained antimicrobial agent release while demonstrating low toxicity to human cells (18). Their potential synergistic effects with conventional antibiotics further emphasize their significance in the ongoing battle against infectious diseases. While FeNPs present numerous advantages, it is essential to consider their application carefully, ensuring they meet safety and efficacy standards for various medical and clinical settings, as underscored by previous research (18). These findings collectively highlight the importance of FeNPs as a novel and versatile class of

antimicrobials with the potential to reshape treatment approaches to combat infectious diseases effectively.

The TEM analysis presented in Figure 2, complemented by the detailed tabulated data, offers a comprehensive exploration of the structural morphology, size, and dispersity of FeNPs under varying conditions, particularly in the presence of chitosan (Cs) and siRNA. These findings provide crucial insights into the characteristics of these modified nanoparticles. Figure 2A and its zoomed-in version (2Ai) provide a glimpse into the structural morphology of the FeNPs in their native state. The nanoparticles appear as smooth, spherical particles, with an average size of 50.6 ± 9.4 nm. This size range, falling between 40 nm to 60 nm, aligns with the anticipated size range for iron nanoparticles and is consistent with previous research (19). This conformity with existing literature reinforces the success of the FeNP synthesis. However, the narrative takes an intriguing turn when we analyse Figure 2B and its zoomed counterpart (2Bi). The introduction of chitosan alongside FeNPs results in a notable change in particle size and dispersity, with FeNPs now forming clusters while maintaining their spherical shape. The average size increases to 95.2 ± 17.5 nm, encompassing a size range of 77.7 nm to 110 nm. This increase in size and the formation of clusters under the influence of chitosan is consistent with prior studies examining the aggregation of nanoparticles in the presence of polymers (20). These findings underscore the complex behavior of nanoparticles and their response to the introduction of modifying agents, aligning with established knowledge in the field. Finally, Figure 2C demonstrates the TEM analysis of FeNPs successfully conjugated with chitosan and siRNA, resulting in a composite structure. These composite nanoparticles exhibit an average size of 117.3 ± 11.9 nm, spanning a size range from 105.4 nm to 129.2 nm. This enlarged size compared to FeNPs alone underscores the incorporation of chitosan and siRNA, in line with prior research on siRNA-loaded nanoparticles for gene delivery (21). These results, when viewed through the lens of previous literature, confirm the expected behaviour of iron nanoparticles and their modifications. The size and morphology of FeNPs in their native state are in line with established references (22), validating the success of

FeNP synthesis. The significant increase in particle size and the formation of clusters in the presence of chitosan (Figure 2B) are in accordance with previous research highlighting the impact of polymers on nanoparticle aggregation (23). This has substantial implications for understanding how these modified nanoparticles might behave in biological environments and in drug delivery systems. The TEM analysis further substantiates the successful conjugation of FeNPs with chitosan and siRNA (Figure 2C), a key development in the realm of targeted therapies and drug delivery, consistent with prior investigations into siRNA-loaded nanoparticles for gene delivery (24).

The selection of the *AP-65* gene as the target for the *T. vaginalis* SIRNA2 (5'-gttccagatc tcaagaacca g-3) siRNA in our study was underpinned by its pivotal role in the pathogenesis of *T. vaginalis* (25). The *AP-65* gene encodes a protein associated with adherence to host cells, a fundamental process in the establishment and maintenance of infection by this parasite. Existing literature underscores the significance of *AP-65* in promoting host-parasite interactions and, consequently, the persistence of *T. vaginalis* infections (26). By specifically targeting this gene, our approach aimed to disrupt the critical molecular pathways associated with adhesion, potentially hindering the parasite's ability to establish infection. The use of *T. vaginalis* SIRNA2, tailored to the *AP-65* gene, thus holds promise as a strategy to mitigate the virulence of *T. vaginalis*.

The band shift assay data in reference to previous studies (27), offer insights into the critical conditions governing the effective binding of functionalized nanocomplexes with siRNA. In Figure 3, the positive control confirmed that siRNA, when unbound to nanoparticles, exhibited mobility through the agarose gel, consistent with established observations (28). Conversely, the negative control, featuring nanoparticles alone, displayed no siRNA bands, confirming that nanoparticles did not trap siRNA, a phenomenon consistent with findings in related research (20). According to the analysis, with increasing nanoparticle volumes, migrating siRNA bands were observed, indicating incomplete binding, a phenomenon described in prior studies on nanoparticle-siRNA complexes (29). Additionally, with further increased nanoparticle volumes, no migrating siRNA bands were observed,

as shown in similar studies (20). Lane 6 of Figure 3 stood out as the epitome of optimal binding, suggesting that at this specific nanoparticle-to-siRNA ratio, effective binding occurred, preventing siRNA mobility through the gel. This is a pivotal finding. These results collectively provide crucial insights into the design and optimization of siRNA delivery systems, granting precise control over nanocomplex formulations to achieve the desired level of siRNA binding efficiency. Such a nuanced understanding of siRNA binding efficiency is instrumental in advancing therapeutic applications, particularly in the context of gene silencing and targeted therapies within the realm of precision medicine and personalized treatments, an area rich in potential as indicated by prior research (30).

The results from the *RNase A*-mediated digestion study, discussed in relation to earlier studies (31), offers a profound glimpse into the protective capabilities of nanocomplexes formed with siRNA, a critical aspect in gene silencing and targeted therapeutic applications, especially in the face of enzymatic challenges. In this study, nanocomplexes were established using siRNA at various mass ratios, determined beforehand by the band shift assay. Lane 1 of Figure 4, the negative control, revealed that in the absence of any nanoparticles and solely under the influence of *RNase A*, siRNA suffered complete degradation, as demonstrated by the absence of any discernible bands on the gel. This vividly underscores the potent enzymatic threat posed by *RNase A* (32). In contrast, the positive control in Lane 2, where siRNA was treated with nanoparticles but not with *RNase A*, presented a distinct migrated band, indicating that the nanoparticles alone did not degrade the siRNA. This affirms their non-destructive nature in the absence of the enzyme, which corresponds with prior research findings (33). The most intriguing observations, however, lie in Lanes 1 to 3. These lanes featured nanoparticles introduced at volumes ranging from 0.001 to 0.003 microliters, and contrary to expectations, siRNA bands were still visible. These unexpected bands suggested that the nanoparticles effectively shielded the siRNA from *RNase A*-mediated degradation, ensuring the siRNA's integrity and visibility on the gel. This finding underscores the nanoparticles' capability to safeguard siRNA against enzymatic threats and adds an exciting dimension to their potential for preserving siRNA integrity in challenging

biological environments. The fact that these bands appear in the presence of *RNase A* emphasizes the nanoparticles' promise in gene silencing and targeted therapeutic applications when confronted with enzymatic challenges, reinforcing their potential to enhance the stability and effectiveness of siRNA-based therapies, in accordance with previous studies (33).

Figure 5 describes the effects of iron nanoparticles, chitosan, and siRNA on the viability of vaginal epithelial cells, a topic of great importance in the context of women's health. The concentration-dependent impact observed in this study aligns with existing literature, which underscores the pivotal role of nanoparticle concentrations in shaping their biological responses (34). At a high concentration of 1000 µM, we observed a decrease in cell viability, indicating some toxicity. This outcome aligns with previous findings where nanoparticles have exhibited cytotoxicity at elevated concentrations (35). However, as the concentration decreased, a distinct shift in the effects was observed. At 500 µM and 250 µM, cell viability improved, suggesting potential stimulatory effects, similar with earlier studies where nanoparticles have been found to promote cell growth and proliferation (36). Subsequent concentrations of 125 uM, 62.5 uM, and 31.25 uM maintained stable cell viability, indicating a potential "safe zone" for these nanoparticles. Such intermediate concentrations are often associated with the maintenance of cell health in nanoparticle toxicity studies (37). At a lower concentration of 15.625 uM, cell viability further increased, possibly indicating a continued stimulatory response, in line with documented positive effects of nanoparticles on cell proliferation (36). Conversely, at the lowest concentration tested, 7.8125 uM, a minor decrease in cell viability was observed, consistent with previous findings suggesting that very low nanoparticle concentrations may have detrimental effects on cells (38). These findings collectively underscore the importance of considering nanoparticle concentrations when assessing their effects on cell viability, providing valuable insights into the biocompatibility and potential stimulatory effects of these nanoparticles. This information forms a solid foundation for the development of strategies for drug delivery and therapeutic interventions in

the field of women's health, where tailored approaches are in high demand due to the complexities and intricacies of the female physiology (39).

Figure 6 provides critical insights into the interactions between siRNA, nanocomplexes, and *T. vaginalis*, shedding light on the potential formation of complexes or binding events. Lane 2, featuring siRNA alone, shows the expected migration pattern, with siRNA molecules migrating beyond the 100bp DNA ladder, as in accordance with their small size. However, it's in Lanes 3 and 5 where the most intriguing findings emerge. In these lanes, where siRNA was combined with nanocomplexes and samples of *T. vaginalis*, represented by sample 253 and sample 270, respectively, a striking alteration in migration behaviour is observed. In both cases, the mobility of siRNA is significantly reduced to approximately half of that observed for siRNA alone (as seen in Lanes 2 and 4). This dramatic change strongly suggests a successful interaction between the siRNA, nanocomplexes, and *T. vaginalis* samples, indicating the formation of complexes or binding to the *T. vaginalis* DNA (29).

These findings are of particular importance in the context of siRNA-based therapeutics and *T. vaginalis* research. The reduced mobility of siRNA in the presence of *T. vaginalis* samples strongly implies a binding interaction between the siRNA and the pathogen's genetic material. This insight could be invaluable for the development of novel strategies for the treatment of *T. vaginalis* infections using siRNA-based approaches. It also underscores the potential for using nanocomplexes to facilitate the delivery of siRNA to *T. vaginalis*, a concept previously explored in the field of nanotechnology-based drug delivery (40). Additionally, these results align with the growing body of research focusing on the use of siRNA as a tool for targeted genetic interventions, highlighting its versatility and potential for further studies in gene silencing and the development of novel therapeutics (41).

The results presented in Figure 7 provide valuable insights into the complex interplay between iron nanocomplexes, *T. vaginalis*, and vaginal epithelial cell viability, shedding light on the potential for novel therapeutic approaches and the mechanisms underlying these interactions. The concentration-

dependent shift in cell viability is a central theme in this study and carries significant implications. At the highest concentration of 1000 µM, the combination of iron nanocomplexes with *T. vaginalis* resulted in a substantial reduction in cell viability, with the percentage dropping to approximately 51.58%. This observation suggests a considerable cytotoxic effect at high concentrations, likely associated with the presence of *T. vaginalis* and its adherence to the vaginal epithelial cells. The cytotoxic effects of *T. vaginalis* on host cells have been well-documented in the literature (42), and the current findings underscore the potential for iron nanocomplexes to exacerbate this cytotoxicity at elevated concentrations.

As the concentration of iron nanocomplexes decreases to 500 µM and 250 µM, there is a progressive improvement in cell viability, reaching around 68.64% and 84.92%, respectively. This shift indicates a reduction in cytotoxicity, highlighting the dynamic nature of the interactions at different concentrations. However, the most interesting finding arises at 125 µM, where cell viability remarkably increases to almost 99.35%. This marked recovery in cell viability suggests that the siRNA used successfully silenced the adhesion gene, preventing *T. vaginalis* from adhering to vaginal epithelial cells, thereby sparing them from cytotoxicity. This result has significant implications for potential therapeutic strategies aimed at preventing *T. vaginalis* infection and its associated cytotoxic effects. Subsequent concentrations, ranging from 62.5 µM to 15.625 µM, continue to demonstrate high cell viability, with values approaching or exceeding 99%. These findings open the door to the development of tailored approaches for managing *T. vaginalis* infections and related cytotoxicity, with potential applications in reducing the burden of *T. vaginalis* induced vaginal infections. However, at the concentration of 7.8125 µM, there is a noticeable decrease in cell viability to approximately 65.84%. This result is particularly significant as it suggests that at this specific concentration, the combination of iron nanocomplexes with *T. vaginalis* may have a cytotoxic effect on vaginal epithelial cells, indicating that the relationship between nanocomplexes and *T. vaginalis* is complex and concentration-dependent (43). The control condition at 0 µM, representing *T. vaginalis* treatment

alone, confirms the expected baseline cell viability of 20%. In this scenario, where the adhesion gene silencing is not in effect, *T. vaginalis* effectively infects the vaginal epithelial cells and induces substantial cell death, in line with existing knowledge of *T. vaginalis* pathogenicity. These findings provide a foundation for further investigations into the potential of siRNA-mediated gene silencing as a therapeutic strategy against *T. vaginalis* infection and cytotoxicity, as well as the concentration-dependent effects of iron nanocomplexes. The results underscore the dynamic and multifaceted nature of these interactions and their relevance for the development of novel therapeutic interventions in the context of *T. vaginalis* infections. This research builds upon and aligns with previous work exploring the interactions between nanomaterials, pathogens, and host cells in the context of infectious diseases (44).

Conclusion and Limitations

Our research provides a comprehensive foundation for the development of nanocomplex-based siRNA delivery systems, the exploration of innovative approaches to combat *T. vaginalis* infections, and the advancement of nanotechnology in the field of precision medicine and personalized treatments. These findings expand our knowledge and offer promising prospects for the future of nanomedicine, with potential applications in drug delivery systems, gene silencing, and targeted therapies across various biomedical domains. The multifaceted nature of nanocomplexes and their concentration-dependent effects underscore the importance of further research in harnessing the full potential of these materials to address complex challenges in healthcare and biotechnology. While our research contributes significantly to the field, it is important to acknowledge its limitations. First, our experiments were primarily conducted in controlled laboratory settings, and the transition of these findings to more complex *in vivo* environments may necessitate further studies. Additionally, as our results indicate concentration-dependent effects of nanocomplexes on cell viability, precise dosage control and extensive pharmacokinetic investigations will be required to ensure the safety and efficacy of these delivery systems. Moreover, our study primarily focuseed on the cellular aspects of siRNA delivery

and protection, leaving room for further research to explore intracellular mechanisms, long-term effects, and potential off-target effects of nanocomplexes in gene silencing and targeted therapies. These limitations highlight the need for ongoing research to unlock the full potential of nanocomplexes and to address the multifaceted challenges in healthcare and biotechnology comprehensively.

Conflicts of Interest

The authors declare no conflict of interest.

Funding: National Research Foundation, College of Health Sciences funding (University of KwaZulu-Natal)

Ethical Standards

Ethical approval was obtained from the Biomedical Research Ethic Committee (BREC) (BREC/00003557/2021).

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CHAPTER 6:

Discussion and Conclusion

Metronidazole resistance in *Trichomonas vaginalis* represents a critical and evolving issue in the realm of public health (Paulish-Miller *et al.,* 2014). The emergence of resistance to metronidazole, a commonly prescribed drug for treating trichomoniasis, threatens to undermine the effectiveness of this essential medication (Meri *et al.,* 2000). As the first line of defence against *T. vaginalis* infections, metronidazole has long been the cornerstone of treatment protocols. However, the increasing incidence of resistant strains of *T. vaginalis* has raised alarms, highlighting the urgent need for innovative strategies to address this complex challenge (Schwebke & Barrientes, 2006).

Trichomoniasis, caused by *T. vaginalis*, is one of the most prevalent non-viral sexually transmitted infections worldwide, affecting millions of individuals each year (Mabaso & Abbai, 2021). Metronidazole, with its antimicrobial properties, has played a pivotal role in mitigating the impact of this parasitic infection (Cudmore *et al.,* 2004). Nevertheless, the emergence of metronidazole resistance jeopardizes the efficacy of this trusted treatment and necessitates a re-evaluation of our approach to combating the pathogen. The implications of metronidazole resistance are profound, potentially leading to prolonged and recurrent infections, increased healthcare costs, and heightened risks of transmission (Cudmore *et al.,* 2004). In response to this pressing concern, the aims and objectives of the present thesis converge to form a comprehensive strategy to understand and tackle metronidazole resistance in *T. vaginalis*. The research endeavours to uncover the roles played by iron, in the form of nanoparticles derived from *Ocimum tenuiflorum*, and *T. vaginalis* viruses in the development of metronidazole resistance. Moreover, the objectives of this work encompass the investigation of iron metabolism, the association between *T. vaginalis* viruses and drug resistance, and the exploration of medicinal plants as potential therapeutic options. The synthesis of iron nanoparticles and their utilization for siRNA-based targeted gene delivery further expand the arsenal of innovative approaches to counter metronidazole resistance. The discussion within this thesis aims to provide a

holistic understanding of the research findings and how they align with these objectives and aims. It not only elucidates the scientific discoveries presented in the preceding sections but also underscores their significance in the context of combating metronidazole resistance. This body of work represents a multi-faceted approach to address the challenges posed by drug-resistant *T. vaginalis* isolates and offers a promising array of potential solutions. By understanding the complex interplay of iron nanoparticles, gene silencing through siRNA, the role of specific genes like *AP-65*, and the influence of *T. vaginalis* viruses, the research presented in this thesis contributes significantly to the ongoing efforts to redefine treatment approaches and ensure the continued effectiveness of metronidazole in managing *T. vaginalis* infections.

Iron metabolism is a fundamental component of the thesis, and its intricate connection with metronidazole resistance in *T. vaginalis* is a key focal point. The research aims to delve into the role of iron metabolism and how it influences both drug resistance and gene expression within the parasite. By doing so, it offers novel insights and potential strategies for overcoming this growing challenge in the treatment of trichomoniasis. The investigation into iron nanoparticles derived from *Ocimum tenuiflorum* opens up exciting possibilities in combating metronidazole resistance. These nanoparticles have demonstrated their ability to disrupt biofilms and generate reactive oxygen species (ROS), which are crucial factors in addressing drug resistance (Makabenta *et al.,* 2021). Metronidazole relies on ROS to inflict damage on the DNA of *T. vaginalis*, making alterations in iron metabolism a critical determinant of drug efficacy (Argáez-Correa *et al.,* 2019). By studying the effects of these iron nanoparticles, this research provides a potential avenue for enhancing the efficacy of metronidazole against resistant isolates. One of the noteworthy aspects of the research is the versatility of iron nanoparticles. They can be modified through conjugation with chitosan and siRNA, a feature that holds great promise for more targeted therapeutic interventions (Babu *et al.,* 2016). The demonstrated success in conjugating iron nanoparticles with chitosan and siRNA is particularly noteworthy as it aligns with prior investigations into siRNA-loaded nanoparticles for gene delivery (Kamalzare *et al.,*

2022). This opens up the possibility of utilizing these nanoparticles as carriers for specific therapeutic agents, which can be directed precisely to their intended targets.

The study also goes beyond the laboratory setting by observing the behavior of these nanoparticles within biological environments and drug delivery systems. The increase in particle size and the formation of clusters, as shown in Figure 2B, exemplify the nuanced behaviour of nanoparticles when introduced alongside modifying agents (Yusuf *et al.,* 2023). Understanding this behaviour is crucial as it aids in predicting how these nanoparticles would behave in real-world applications, which is invaluable when considering their utilization in clinical or therapeutic settings. The role of *T. vaginalis* viruses in the context of metronidazole resistance represents another crucial dimension of this research. The formation of complexes or binding events between siRNA, nanocomplexes, and *T. vaginalis*, as indicated in the results, suggests that siRNA-mediated gene silencing holds substantial therapeutic potential (Graves *et al.,* 2019). These findings open the door to the concept of using siRNA for targeted gene delivery within the context of *T. vaginalis* infections. By silencing specific genes, such as *AP-65*, the research aims to mitigate the virulence of *T. vaginalis*, as *AP-65* plays a pivotal role in promoting host-parasite interactions and, consequently, the persistence of *T. vaginalis* infections (Mundodi *et al.,* 2004). The interplay between siRNA, nanocomplexes, and the parasite in Figure 6 alludes to the possibility of developing precise interventions to disrupt the life cycle of *T. vaginalis*. By specifically targeting genes crucial for the parasite's persistence and pathogenesis, the research offers a glimpse of a potential therapeutic approach that may be more effective and precise than conventional treatments.

The investigation into medicinal plants as potential therapeutics against *T. vaginalis* is a crucial facet of this research, aligning with the objective of exploring alternative treatment options to tackle metronidazole resistance. It also considers the diverse intricacies of *T. vaginalis* infections and aims to contribute to a more comprehensive approach to managing this pathogen. One of the significant findings in this research is the concentration-dependent effect of iron nanoparticles on the viability of vaginal epithelial cells, which serves as a model system for studying the impact of potential therapeutic

agents (Naqvi *et al.,* 2010). This observation is pivotal as it can inform the development of new treatment strategies that not only target the parasite but also consider the health of the host's cells. At high concentrations, as seen in the research, iron nanoparticles display cytotoxicity, which is consistent with findings from earlier studies (Wei *et al.,* 2021). This cytotoxic effect at elevated concentrations is a valuable observation as it underscores the importance of carefully considering the dosage and concentration when employing these nanoparticles as therapeutic agents. It emphasizes the need for balancing the therapeutic effect on *T. vaginalis* with the safety and well-being of the host's cells. This information is of paramount importance when designing treatment regimens and ensures that the potential therapeutic agents do not inadvertently harm the host. However, the most intriguing and promising aspect is the response observed at lower concentrations. At concentrations such as 125 µM and even down to 7.8125 µM, the iron nanoparticles demonstrate potential stimulatory effects on cell viability (Santhoshkumar *et al.,* 2023). These findings indicate that, at specific concentrations, the nanoparticles may promote the growth and proliferation of vaginal epithelial cells. This observation is particularly encouraging as it suggests that iron nanoparticles could have dual benefits; they could combat *T. vaginalis* while also supporting the health of the host's cells. The concentration-dependent effects of iron nanoparticles provide valuable insights into the fine-tuning of therapeutic strategies. Researchers and clinicians can use this information to design treatment protocols that optimize the beneficial effects of these nanoparticles on host cells while effectively targeting *T. vaginalis*. This nuanced approach is particularly relevant in the context of women's health, where the intricacies of the female physiology require tailored interventions (Yuksel, 2011). The synthesis of iron nanoparticles and their combination with siRNA-based gene delivery holds significant promise as a strategy to combat metronidazole resistance in *T. vaginalis*. These findings from the research demonstrate the multifaceted potential of these nanocomplexes in reshaping the approach to treatment of *T. vaginalis* infections.

Iron nanoparticles are particularly intriguing due to their antimicrobial properties and biocompatibility. The research findings highlight that they can be engineered for biocompatibility and sustained antimicrobial agent release while maintaining low toxicity to human cells (Arias *et al.,* 2018). This makes them a compelling candidate for further development as antimicrobial agents in the context of *T. vaginalis* infections. The significant increase in particle size and the formation of clusters when introduced alongside chitosan, as shown in Figure 2B, provides insights into how polymers influence nanoparticle aggregation (Moraru *et al.,* 2020). Understanding the behaviour of these modified nanoparticles in biological environments and drug delivery systems is crucial, as it aids in predicting their effectiveness in targeted therapy.

SiRNA emerges as a valuable tool for targeted gene delivery in *T. vaginalis*. The band shift assay and *RNase A*-mediated digestion study showcase the efficiency of nanocomplexes in protecting siRNA from enzymatic degradation, a pivotal aspect of gene silencing and targeted therapeutic applications, especially in the presence of enzymatic challenges (Oliveira *et al.,* 2006). These findings highlight the robustness of nanocomplexes in preserving siRNA integrity in challenging biological environments, further strengthening the potential of siRNA-based therapies in gene silencing and the development of novel therapeutics (Xue *et al.,* 2014). The successful interaction observed between siRNA, nanocomplexes, and *T. vaginalis* underscores their capacity to withstand the enzymatic challenges often faced in biological systems. This further solidifies the promise of siRNA-based therapies for targeted interventions in *T. vaginalis* infections. The concentration-dependent effects of iron nanoparticles on vaginal epithelial cells provide a critical insight into the complexity of using these nanoparticles in therapeutic strategies. The research findings emphasize the necessity for careful optimization of the dosage when employing these nanoparticles as potential therapeutics. High concentrations, as indicated, may lead to cytotoxicity, which aligns with previous research (Naqvi *et al.,* 2010). This observation underscores the importance of balancing the therapeutic effects on *T.*

vaginalis with the safety and well-being of the host's cells, ensuring that the therapeutic agents do not inadvertently harm the host.

Conversely, the stimulating effect observed at lower concentrations, particularly at 125 µM and below, is a promising finding. This suggests that, at specific concentrations, iron nanoparticles may not only combat *T. vaginalis* but also promote the growth and proliferation of vaginal epithelial cells. This dual benefit is particularly encouraging, as it signifies that iron nanoparticles could play a vital role in both addressing the infection and aiding in the recovery of host cells. Therefore, careful consideration of the concentration of these nanoparticles in therapeutic strategies is essential to maximize their efficacy and safety. The research findings presented in this thesis provide a compelling insight into the potential of alternative therapeutics for addressing metronidazole resistance in *T. vaginalis*. The multifaceted approach encompassing iron nanoparticles, siRNA-based gene delivery, and medicinal plants offers a ray of hope for more effective, precise, and innovative treatments.

Iron nanoparticles, as demonstrated in the research, exhibit antimicrobial properties and biocompatibility, making them a promising candidate for combating *T. vaginalis* infections. Their ability to disrupt biofilms, generate ROS, and potentially overcome metronidazole resistance aligns with the critical need for novel therapeutic strategies (Vallet-Regí *et al.,* 2019). Furthermore, the capacity to modify iron nanoparticles through conjugation with chitosan and siRNA provides a gateway to more targeted therapeutic interventions (Herdiana *et al.,* 2021). These findings not only align with the objectives of the thesis but also add to the growing body of knowledge in the battle against metronidazole resistance in *T. vaginalis*. They create opportunities for further research and therapeutic innovations that have the potential to redefine the treatment landscape for *T. vaginalis*. SiRNA-based gene delivery, another pivotal component of the multifaceted approach, emerges as a potent tool for targeted interventions. The band shift assay and *RNase A*-mediated digestion study illustrated the efficiency of nanocomplexes in protecting siRNA from enzymatic degradation, a crucial element in gene silencing and targeted therapeutic applications (Haupenthal *et al.,* 2006). The

successful interaction between siRNA, nanocomplexes, and *T. vaginalis* reinforces their potential to preserve siRNA integrity in challenging biological environments, thereby enhancing the potential of siRNA-based therapies in gene silencing and the development of novel therapeutics (Fernando *et al.,* 2018).

The exploration of medicinal plants as therapeutics against *T. vaginalis*, as highlighted in this research, brings an essential natural element into the alternative therapeutic approach. The concentration-dependent effects of iron nanoparticles on the viability of vaginal epithelial cells indicate that careful dose optimization is necessary to balance therapeutic efficacy and host cell safety (Soetaert *et al.,* 2020). This dual potential of iron nanoparticles to combat *T. vaginalis* while also promoting the growth of vaginal epithelial cells is particularly encouraging and emphasizes the importance of fine-tuning the treatment strategy. Collectively, these alternative therapeutic approaches not only have significant potential in addressing metronidazole resistance in *T. vaginalis* but also pave the way for a new era of research and innovation in this field. They align with the aims and objectives of the thesis by shedding light on the roles of iron, *T. vaginalis* viruses, and medicinal plants in metronidazole resistance. Moreover, they contribute to the broader body of knowledge surrounding *T. vaginalis* treatment and metronidazole resistance. These findings open doors to future investigations, therapeutic developments, and a redefined landscape for addressing *T. vaginalis* infections.

Conclusion

The thesis discusses the critical issue of metronidazole resistance in *T. vaginalis*, a pressing concern in public health. Resistance to metronidazole for cases of trichomoniasis is on the rise. This raises concerns about the effectiveness of treatment and calls for innovative solutions. Medicinal plants are considered as therapeutics, but dose optimization is essential to balance therapeutic effectiveness and host cell safety. Iron metabolism, particularly involving iron nanoparticles derived from *Ocimum tenuiflorum*, have the potential to disrupt biofilms, generate reactive oxygen species, and improve metronidazole efficacy. In addition, the role of siRNA-based gene delivery as tools for targeted

interventions. offers potential to reduce *T. vaginalis* virulence. These findings contribute to public health efforts to manage this challenging infection.

Limitations

- The research may be limited by a relatively small sample size, potentially affecting the generalizability of the findings to broader populations of *T. vaginalis* isolates.
- The study primarily focuses on *in vitro* experiments, which may not fully replicate the complex *in vivo* conditions that occur within a host. This could affect the translation of the results to real-world clinical scenarios.
- The study's duration was limited, and longer-term observations could provide a more comprehensive understanding of metronidazole resistance dynamics.
- The research did not consider potential interactions between metronidazole and other medications or treatment regimens that individuals may be receiving, which could impact the effectiveness of metronidazole in real-world cases.
- The specific isolates, conditions, and methodologies used in the study does not fully represent the diversity of real-world *T. vaginalis* infections, limiting the broad applicability of the findings.

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APPENDICES

Appendix A

Biomedical Research Ethics Committee Approval (UKZN)

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

04 January 2022

Mr Rowen Govender (215023500)
School of Clinical Medicine
Medical School

Dear Mr Govender,

Protocol reference number: BREC/00003557/2021
Project title: Identifying mechanisms associated with metronidazole resistance in Trichomonas vaginalis and investigating newer therapeutics against this pathogen
Degree: PhD

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 04 January 2022. Please ensure that any outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is subject to national and UKZN lockdown regulations, see (http://research.ukzn.ac.za/Libraries/BREC/BREC_Amended_Lockdown_Level_1_Guidelines.sflb.ashx). Based on feedback from some sites, we urge PIs to show sensitivity and exercise appropriate consideration at sites where personnel and service users appear stressed or overloaded.

This approval is valid for one year from 04 January 2022. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2020) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 08 February 2022.

Yours sincerely,

[redacted]

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

Founding Campuses: Edgewood, Howard College, Medical School, Pietermaritzburg, Westville

INSPIRING GREATNESS

Appendix B

Diamond's TYM Medium preparation (1 Litre)

20g	BBL™ trypticase peptone
10g	Yeast extract
5g	Maltose
1g	L-cysteine hydrochloride
0.2g	L-ascorbic acid
0.5g	Agar
900ml	Distilled water
100ml	Heat inactivated donor horse serum

All the powder components were dissolved in 900ml distilled water and adjusted to a pH of 6.0. The medium was then autoclaved at 121^{o}C for 15 minutes then cooled to 50^{o}C in the water bath. Donor horse serum was heat inactivated at 56^{o}C for 30 minutes then added into Diamond's TYM medium. The medium was then aliquoted into 5ml in polystyrene 15ml tubes and stored at 2-8^{o}C.

Antimicrobials were not added in the medium that was to be used for susceptibility testing. If contamination was observed in cultures then amikacin (4µg/ml), amphotericin B (5µg/ml), ampicillin (1mg/ml), chloramphenicol (1µg/ml), ciprofloxacin (2µg/ml) and vancomycin (5µg/ml) was added intothe medium during preparation.

T. vaginalis scoring criteria

Table 1 was used to score *T. vaginalis* growth and motility after 48 hours of incubation. The MIC wasthe lowest metronidazole concentration with a score of +1.

Table 1: Scoring of *Trichomonas vaginalis* growth and motility (Upcroft and Upcroft, 2001)

Score	Interpretation
1+	0-10 motile parasites; not more than 20% coverage of well surface
2+	> 10 motile parasites; 20% to 50% coverage of the well surface
3+	> 50 % coverage of the well surface = almost confluent growth with much motility
4+	confluent growth with full motility

Inoculum preparation and standardization

• Each T. vaginalis isolate was cultivated in 5ml drug-free Diamond's TYM medium and incubated at 37oC and sub-cultured at 24 to 48 hour intervals until axenic non-contaminated cultures were obtained.

• For the inoculum preparation, 500 µl of T. vaginalis culture was added to 5 ml of fresh pre- warmed Diamond's TYM medium and incubated at 37oC.

• After 24 hours of incubation, 1ml of the culture was transferred into a 15 ml sterile then mixed by gently inverting the tube 3 to 4 times.

• A drop of the transferred culture was placed onto a Neubauer haemocytometer to count the number of viable trophozoites.

• Viability of the trophozoites was assessed by motility, flipping flagella and/or undulating membrane using a light microscope at x400 magnification.

• The number of viable trophozoites per millilitre was then calculated using the following formula:

$$\text{Concentration} = \text{Number of trophozoites/ squares counted} \times 10^4$$

The desired inoculum of 1.5×10^4 and 1×10^5 trichomonads/well for the anaerobic and aerobic assays, respectively was obtained by dilution with antibiotic free pre-warmed Diamond's medium.